NATIONAL INSTITUTE OF CHILD HEALTH AND HUMAN DEVELOPMENT

INTRAMURAL RESEARCH PROGRAM

ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR

1988

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Cover photo
Stages of *Xenopus laevis* embryogenesis,
an important model of vertebrate development.
(Courtesy of Dr. Igor Dawid)

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The Intramural Research Program is broadly concerned with the biological and neurobiological, medical, and behavioral aspects of normal and abnormal human development. In addition to four major clinical research and training programs in the areas of genetics and endocrinology, a diversity of developmental models are under study in twelve fundamental research Laboratories, drawing upon observations in bacteria, Drosophila, yeasts, viruses, molluscs, frogs, rodents, and subhuman primates. Disciplines employed in these studies include biochemistry, virology, molecular biology, immunology, pharmacology, genetics, cell and neuronal biology, biophysics, mathematical and theoretical biology, reproductive physiology, and comparative ethology. During the past year, we have emphasized a number of research programs that we believe have a particular promise and timeliness. In these areas, new resources have been added so that the investigators will be optimally poised to exploit their recent findings. These programs address problems in the cellular and molecular biology of development (growth and differentiation), wherein we anticipate significant results with broad implications for biomedical research. Indeed, it is our notion that the central, and arguably the most exciting, question in contemporary biology is: What are the mechanisms by which gene expression is developmentally regulated?

For several years, the Laboratory of Molecular Genetics (LMG) has been concerned with understanding the genetic regulation of embryonic development, using several different prokaryotic and eukaryotic models. The tools of contemporary biology, in particular recombinant DNA and monoclonal antibody technology, have greatly accelerated progress in this field. One group, studying the genetic control of ectoderm differentiation in Xenopus (a frog), is searching the upstream region of the epidermal keratin gene for sequences that control tissue specificity. Their general location has been found; the promoter region has been partially mapped and an enhancer found that is specific to the keratin promoter. The group has also studied mesoderm induction in explants of Xenopus laevis embryos, and in collaboration with a group in NCI, progress has been made specifically in characterizing a Xenopus cell line growth factor which induces mesodermal but suppresses ectodermal markers. The study of the molecular control of embryogenesis depends on the identification of suitable marker genes for cell-specific functions. In the past, the LMG has been very successful in isolating many such genes, and progress continues, with markers for Xenopus dorsoventral axis development, for the developing nervous system, and for notochord differentiation being added to the gene library. Genetic regulation of development is also being studied in the fruitfly, Drosophila, with particular attention to homeotic genes, which influence segmentation and segment morphology and appear to be of general importance in eukaryotes since they have also been found in mammalian cells. The proteins encoded by a maternal homeotic gene, fs(1)h, which controls segment identity, have been partly sequenced (from cDNA) and exhibit unusual amino acid sequences, some being homopolymeric runs, such as polyglutamine, and one region which may be sufficiently hydrophobic to be a transmembrane domain. Another homeotic gene being studied is the trithorax (trx) gene, which interacts with fs(1)h, and this year with the aid of trx cDNA, progress was made in identifying the encoded proteins. A new LMG group is identifying hitherto unknown genes involved in segment identity in Drosophila, and is in the process of cloning their DNAs. To understand the molecular mechanisms of such integrative genetic systems, use is made of the fact that they also exist in much simpler organisms, for example prokaryotes and the unicellular eukaryote, yeast. The prokaryote E. coli responds to its nutritional state by regulating the level of the nucleotide, ppGpp. This year much progress was made in unravelling the complex genetic interactions between two operons involved in the synthesis and degradation of ppGpp. Another group is looking at the translational control of synthesis of a yeast transcriptional activator protein, GCN4, which is the proximal regulator of a global cascade of genetic loci controlling the synthesis of amino acids. Progress has been made in mapping the mRNA leader for this protein, which is where translational control appears to reside. The positive and negative regulators of GCN4 synthesis have also been isolated and sequenced. Mutations in these genes have demonstrated that the regulators also act at the translational level. One such regulator, GCD2, a protein kinase, exerts its regulatory effect on GCN4 translation through its kinase activity. Other LMG scientists are investigating the mechanisms, in particular recombination and transcription, by which bacteriophages switch between the lysogenic and lytic states. The primary structures of "attachment sites" in HK022, a coliphage related to the well-known lambda phage, and of proteins involved in host-virus recombination have been determined, and by splicing these elements with those of lambda, it has been possible to locate the critical determinants of phage specificity. Another focus of the research is on the mechanism whereby phages can inhibit superinfection by close relatives. The group has identified an HK022 gene called nun, which codes for a terminator capable of interfering with lambda's N antitermination protein, thereby inhibiting lambda replication in HK022-infected E. coli. The transgenic mouse continues to be invaluable for the study of a variety of functions which control gene expression temporally and spatially. For example, an LMG group has found that the regulatory long terminal repeat sequence of the HIV virus, when carried by transgenic mice, is expressed primarily in skin macrophages, consistant with clinical inferences that these cells may be the primary source of HIV infection. In a further transgenic AIDS-related project, use is being made of the technique of directing genes of interest to the mammary gland, so that encoded proteins can be isolated from the milk. Work is in progress to direct to the milk the soluble form of the CD4 antigen, a lymphocyte receptor able to bind and neutralize HIV. Recent work by another LMG group on murine leukemia virus has shown considerable relevance to the understanding of retroviruses in general. It was found that cells infected with several retroviruses, including HIV, suppress modification of isoacceptor tRNA; this suppression is probably necessary for the frameshifting that allows expression of the pol gene, encoding reverse transcriptase, and thus pol-dependent viral functions.

The Laboratory of Developmental Pharmacology (LDP) is concerned with the structure and molecular mechanisms of expression of genes which encode the drug-metabolizing enzymes, primarily the class of enzymes known as cytochromes P450, which are heme-containing, membrane-bound proteins, located almost exclusively in the endoplasmic reticulum, catalyzing the oxidation of a vast array of non-polar compounds, such as steroids, fatty acids, prostaglandins, drugs, chemical carcinogens and mutagens and other environmental contaminants. Oxidation by P450 is the so-called phase I metabolic step. Depending on the reactivity and formation rate of the oxidized intermediates, they are either further metabolized with the help of conjugating enzymes (phase II metabolism) to be excreted as innocuous polar products, or they can combine covalently with DNA, a step often associated with chemical carcinogenesis and mutagenesis. The LDP has been responsible for proposing a generally accepted nomenclature for P450 enzymes to remove some of the confusion in that area, and now that the amino acid sequences of many of them are known, to group these enzymes into families depending on their sequence homologies. Grouping them in this fashion has allowed the construction of an evolutionary tree, which shows that the ancestral P450 gene originated some 1.5 billion years ago. The study of phase I and phase II metabolism has particular relevance to the question of genetic variability in the capacity to tolerate environmental stress. Detection of such pharmacogenetic polymorphism is probably one the most promising aspects of this work because it may eventually allow physicians to predict who is at most risk for certain environmentally caused disorders. One such polymorphism is that of P450 aryl hydrocarbon hydroxylase inducibility. High inducibility has been correlated with a 20 to 40 fold increased lung cancer risk for smokers. Working on the $P_1$450 gene, which encodes aryl hydrocarbon hydroxylase, the LDP has identified a group of genes known as the [Ah] gene battery. The battery also contains five other enzymes with coordinate metabolic functions: $P_3$450, NAD(P):menadione reductase, glutathione transferase, aldehyde dehydrogenase and UDP glucuronosyltransferase. All six genes have been cloned, are coordinately activated by the Ah receptor, and induced by polycyclic hydrocarbons and tetrachlorodibenzo-p-dioxin (TCDD, "Agent Orange"). It has been possible to map the upstream regulatory region of the Ah receptor gene by isolating cell clones which lack the Ah receptor or in which the receptor/ligand complex is unable to bind to the chromatin, and to transfect various lengths of the $P_1$450 gene into these mutant cell lines. Work is in progress to clone the Ah receptor gene, a repressor gene called AHN, and other regulatory genes, with the ultimate goal of characterizing different human Ah genotypes. Another cytochrome P450 polymorphism concerns debrisoquine hydroxylase deficiency, a common autosomal recessive trait with an incidence of 5 to 10% in the white population. Poor metabolizers of debrisoquine lack P450db1, which is the enzyme that hydroxylates debrisoquine, and respond to administration of this ß-adrenergic blocking agent and many other drugs, such as the antitussive dextromethorphan, with an acute toxic reaction. Conversely, extensive metabolism has been associated with various cancers. The cDNA from the livers of several such affected individuals has been cloned, and db1 pre-mRNA splicing has been shown to be defective, providing a molecular basis for one of man's most common genetic polymorphisms.

The Laboratory of Developmental Neurobiology (LDN) conducts research into several overlapping aspects of nervous system function, such as the control of neuronal growth and maintenance of function, the mechanisms of neurotransmitter release and action, and the nature of ion channels. Much effort has been devoted to the processes whereby nerve cells grow and are maintained, and to the role neuropeptides and other trophic factors, as well as electrical activity, play in these processes. The neuropeptide called vasoactive intestinal peptide (VIP) is one agent by which neurons can stimulate the glial cell to produce substances needed for their survival. Progress was made in defining the mechanisms whereby VIP can achieve its effect; a trophic protein produced by glial cells in response to VIP stimulation, which enhances neuronal survival, and substrates for VIP-induced phosphorylation, have been identified. Neurotrophic factors have also been sought by placing cDNA from differentiated nerve cells into several types of neuronal culture; three clones have been found which enhance neuronal cell survival in these cultures; they are at present being characterized. Studies on neuronal cell survival have been extended to the mechanism of cell death caused by the envelope protein of HIV, gp120. Monoclonal antibodies against the mouse T4 receptor, as well as pentapeptide sequences related to peptide T (an octapeptide in the external envelope protein of HIV responsible for strain differences), have been shown to be effective antagonists of gp120, which suggests that such peptide drugs may be of therapeutic value against the neurological sequelae of AIDS. Neurotransmitters, which act via the NMDA receptor, in addition to their recognized role as mediators of excitatory synaptic transmission, appear to have a neurotrophic role during certain stages of development, and LDN workers have found that they can enhance neuronal survival during electrical blockade. Another group has been working on the regulation of neural development by electrical activity, specifically on the mechanisms involved in the selection of those neural connections that survive vs. those that do not. It appears that chronic electrical stimulation causes an increase in number and strength of synaptic connections in tissue culture. Significant progress has been made by a group of LDN workers in another area of research which has been the focus of much recent interest, namely the role of excitatory amino acids in neurotransmission and in diseases, such as stroke. Because of a technological improvement in the way various drugs and ions can be applied to cultured nerve cells, a so-called fast perfusion system, this group has been able to do high resolution kinetic studies on modulators of glutamate receptor activity, with the goal of unravelling the complexities of excitatory amino acid action. This year they have mainly investigated the role of one important modulator, the zinc ion, which is abundant in certain areas of the brain. Zinc was found to affect three different parameters. The major excitatory pathway mediated by glutamate was blocked, the NMDA receptor subtype being selectively affected, and not the kainate and quisqualate subtype. The major inhibitory pathway mediated by GABA was antagonized by zinc. Zinc also blocked the transient potassium current, which depresses excitability, thus increasing intrinsic excitability of neurons. Taken together with its effects on magnesium and calcium sensitivity, the results of these studies have allowed formulation of a model to explain the complex mode of action of excitatory amino acid neurotransmitters, which accounts for the diverse effects of various modulators on NMDA receptor activity. Neurotransmitter release can by studied in vitro using nerve terminals isolated from the posterior pituitary without contamination by the postsynaptic membranes. They secrete oxytocin and vasopressin in response to depolarizing stimuli. Various details of the release process have been described this year, such as the function of potassium channels, calcium channels, cyclic GMP and the endogenous inhibitory opiate dynorphin, which binds to kappa opiate receptors on the oxytocin nerve terminals, and is co-released with vasopressin. Work is under way to clone the genes which encode potassium channels in an effort to elucidate their molecular structure. Transmembrane signalling mechanisms which require two input sources have been studied in pinealocytes. When pinealocytes are treated with norepinephrine, full stimulation occurs only if both α_1- and ß-adrenergic receptors are activated; occupation of α_1-receptors results in the activation of protein kinase C and that of ß-adrenergic receptors in the activation of adenylyl and guanylyl cyclases. Advances were made in identifying the biochemical steps of this complex activation process.

The primary goal of the Laboratory of Neurochemistry and Neuroimmunology (LNN) has been to understand the interaction, effected by the neuropeptides, between the central nervous system and the endocrine system. The ACTH/endorphin/MSH family of peptides is the particular focus of this group. These peptides are all derived from a single precursor, pro-opiomelanocortin (POMC), and are formed by differential processing in different parts of the brain and pituitary. The major emphasis of this year's work has been the manner in which they are processed. POMC is converted to ACTH, ß-endorphin and MSH by prohormone converting enzyme (PCE), which is an aspartyl protease. It was shown that MSH and N-POMC 1-49, an adrenal mitogen, are generated from a glycopeptide fragment of POMC by PCE. PCE was shown to be structurally related, but not identical to, cathepsin D, and work continues on the cloning of the PCE gene. PCE was located in the secretory vesicles of the pituitary, the site of POMC cleavage, and in elegant experiments using fusion proteins generated last year from genetic constructs, including deletions of various lengths of the 3' end of the POMC gene, electron microscopic immunohistochemical staining has revealed that the first 25 amino acids at the N-terminus of POMC are necessary to direct the prohormone to the secretory vesicles. In another project it has been shown that, in response to a physiological challenge, such as salt adrenal enucleation, the relative predominance of the various POMC cleavage products changes, so that the mitogen N-POMC 1-49 becomes the major component. Another physiological challenge is salt-loading stress, and it was shown that this stress causes POMC mRNA levels in the anterior pituitary to rise prior to an increase in ACTH levels. The factors mediating this response are arginine vasopressin (AVP) and corticotropin releasing hormone (CRH); CRH has a permissive role in the action of AVP. It was found that the mRNA of only AVP was increased in the pituitary of a salt-loaded animal. The two hormones appear to act through separate transduction signals, CRH through cAMP-dependent protein kinase, and AVP through activation of protein kinase C via phospholipase C and phosphaditylinositide breakdown. The LNN continues its work on the embryonic expression of neuropeptides, particularly POMC, which promotes axon regeneration and adrenal cell growth. POMC expression starts very early in embryonic development, even before the pituitary and other neuropeptidergic systems appear. This finding supports the notion that POMC has a role in the development of the CNS.

The Laboratory of Theoretical and Physical Biology (LTPB) continues to develop and refine computer programs and procedures which enable biomedical researchers to optimize biochemical assays and separations, and to evaluate them statistically. Major theoretical advances were made by one group of workers in the mathematical underpinnings of the algorithm and program FLEXIFIT, which allows analysis of curve families, such as are encountered in bioassays and measurements by ELISA and RIA. In its present form the FLEXIFIT program gives potency estimates with proven standard errors, residual variance and degrees of freedom, without making any strong assumptions about curve shape (except that the curves are smooth). This is particularly important if curves with non-identical shapes are to be compared; such is the case, for example, if the responses of different patients to insulin tolerance tests are compared. The program DETECT, which was developed by this group to put analysis of pulsatile hormone secretion on an objective basis, has been tested and compared with other available programs, and found to provide superior sensitivity/specificity ratios, which are a measure of accuracy. The program DESIGN was developed to optimize complex ligand binding experiments involving multiple ligands and multiple binding sites. Together with LIGAND, a program developed earlier by this group, DESIGN allowed the detection of two classes of binding site for phencyclidine, which are distinct from the already known sigma site, and also the characterization of the latter site. Programs designed to assist in the evaluation of self-monitored blood glucose are being clinically tested. Another group has for some time been instrumental in developing quantitative gel electrophoretic methods, which yield information about size, charge and conformation of macromolecules from the relationship between electrophoretic mobility and gel concentration. Recently, quantitative gel electrophoresis has been applied to subcellular particles, such as viruses and cellular vesicles, using new theoretical and computational approaches to gain information about particle shape and malleability. Apparatus developments have increased the sensitivity of these determinations from the microgram to the nanogram level. A major theoretical advance has made it possible to interpret polydisperse gel patterns, which are frequently encountered when separating subcellular particles by gel electrophoresis. This has allowed a physical characterization of semi-synthetic meningitis immunogen preparations, which may prove useful in the quality control of vaccine production. Advances were also made in the stabilization of 2-dimensional protein maps, with the aim of making such maps comparable between laboratories. Another section is developing separation techniques which rely on detection by mass spectrometry. During the past few years, this group has devoted much effort to the elucidation of calcium metabolism in humans by monitoring plasma calcium levels and excreted calcium after administering stable (as distinct from radioactive) isotopes. Using mathematical models, the group has been able to show that calcium absorption is not age dependent; in osteoporotic women it is the rate of absorption and not absolute absorption which appears to decline. It is now also possible to estimate the size of the rapidly exchanging internal pool of calcium; it correlates well with growth rate, and may be reduced in osteoporotic subjects. A third parameter of clinical significance gleaned from such mathematical analysis, the mean residence time of calcium, correlates closely with skeletal mass, and can therefore be used instead of radioisotopic tracers to determine the latter. In a collaborative study with the Developmental Endocrinology Branch, the group has measured cortisol production rates in patients with Cushing's disease and in normal subjects, using mass spectrometry coupled with thermospray liquid chromatography (ThLC/MS). These are the first measurements of true daily production rates, which are independent of plasma volume and clearance, two significant sources of error in radiotracer measurements. An unexpected finding is that the cortisol production rate in normal subjects is about half that generally thought to be normal, possibly accounting for the symptoms of hypercortisolism in patients on "replacement" therapy after adrenal loss. Technological advances in ThLC/MS techniques have resulted in vastly improved detection of labile materials, such as peptides, and this has been applied to the characterization of autocrine peptides secreted by small cell lung carcinomas.

The efforts of the Human Genetics Branch (HGB) are mainly directed towards understanding the molecular bases of heritable diseases and improving the care of children suffering from such diseases. Disorders of lysosomal storage, such as cystinosis, Salla disease and Niemann-Pick disease, continue to be emphasized, as are glycogen storage diseases. Substantial progress was also made this year in determining the molecular basis for inborn errors of collagen metabolism, such as osteogenesis imperfecta (OI) and related bone diseases. OI is thought to be caused by point mutations in the collagen gene. Five point mutations were detected, two of which have been localized in the 3' end of the α-chain of type I collagen, using a new technique which relies on detecting mismatches between ribonucleotide probes made from cDNA and mRNA made by the mutant alleles in cultured fibroblasts of patients with OI. Prenatal diagnosis of such a crippling disease is of obvious importance. This has not been possible with amniocentesis, because cultured cells from the amniotic fluid exhibit atypical collagen modification. In a first step to making prenatal diagnosis possible, this group has demonstrated that cultured cells from the chorionic villi of pregnancies eventuating in infants with osteogenesis imperfecta show the same defect as the patient's fibroblasts. Progress was also made in identifying a mechanism that could play a role in another devastating heritable disease, cystic fibrosis. An HGB group is studying the basic question of how mucosal epithelia avoid continuous inflammatory responses when they are constantly exposed to foreign antigens. The group has been studying several proteins, induced by endogenous steroids and known to inhibit phospholipase A_2, an enzyme involved in initiating the arachidonic cascade, by which prostaglandins and leukotrienes (mediators of inflammation) are synthesized. They found that two such proteins, rabbit uteroglobin and human lipocortin, have sequence homologies, and that a synthetic nonapeptide, made to correspond to the region of strictest homology, was a potent inhibitor of phospholipase A_2 in vitro and a potent antiinflammatory agent in vivo. This group has now found a uteroglobin-like protein in human tracheobronchial, endometrial and prostatic mucosal epithelium, that may be lacking in patients suffering from cystic fibrosis, who are known to have defective arachidonate metabolism. Success was also achieved in the technology for producing recombinant uteroglobin for site-directed mutagenesis studies and on a scale large enough for purification. Fetal alcohol syndrome (FAS) is one of the major preventable causes of mental retardation in our society. It is interesting that not all mothers who abuse alcohol give birth to babies with FAS. It has been hypothesized that certain individuals and their fetuses have an abnormal transketolase (an enzyme involved in the production of ribose-5-phosphate, a necessary precursor for nucleotide synthesis) which is genetically predisposed to require more thiamine than in unaffected individuals, and that they are therefore more sensitive to the thiamine-deficiency that accompanies alcohol abuse. Progress was made in the production of a prenatal diagnostic screening test for susceptibility to FAS, by showing that amniotic cells derived from women bearing FAS babies are far less able to survive in thiamine-deficient culture medium than cells obtained during normal pregnancy. Other groups in the HGB are studying certain of the factors affecting mammalian differentiation, such as proteins which are developmentally regulated. One such protein is alpha-fetoprotein, made by fetal liver and by hepatomas, and structural differences between AFP mRNA extracted from fetal or adult liver have been found. Rat hepatocytes were used to study factors capable of maintaining differentiation in vitro, such as cAMP, glucocorticoids and retinoic acid. Studies of differentiation in whole animals is made difficult by the relative scarcity of viable mutants. The albino strain of mouse has deletions on chromosome 7, near the albino locus, which also affect the levels of several liver-specific enzymes, leading to the proposal that transacting factors from the albino locus control many liver genes. However, homozygous albino mice die a few hours after birth. Therefore mouse cell lines from both wild-type and albino mice are being established to study these trans-acting factors. Another HGB laboratory is concerned with genetics of phase II detoxification enzymes such as UDP-glucuronosyltransferase. This transferase is coordinately regulated by the Ah gene (see LDP in this summary). An interesting finding is that when a truncated form of the transferase gene, lacking that part which directs insertion of the product into the membrane, is expressed in the cytosol of a yeast cell, the substrate specificity changes. Work is in progress to locate the genetic lesions responsible for the defective glucuronidation in patients with Crigler-Najjar and Gilbert's disease.

The Cell Biology and Metabolism Branch (CBMB) continues its work on cellular and molecular biologic aspects of human iron metabolism, as well as the T cell antigen receptor, interleukin-2 receptor and other receptors involved in the immune response. In addition, a study was initiated during the past year on membrane proteins encoded by the HIV virus. Two sections are studying the control of iron metabolism, which is vital to the health and proliferative ability of all cells; failure of this control is responsible for one of the most common human hereditary diseases, hemochromatosis. Of central importance to the control of cellular iron levels are two proteins, ferritin and the transferrin receptor. The Lab's success of the past few years in demonstrating iron responsive elements with sequence homologies in the mRNAs encoding both ferritin (5') and the transferrin receptor (3') was followed this year by the identification of a protein or proteins capable of iron-dependent binding to both of these elements. The findings enabled the group to formulate a unified model to explain the complex (and inverse) regulation of these two proteins, and evidence has accrued to support the model. At present, studies are being conducted to determine the biochemical mechanism by which the binding protein can respond to iron levels and reversibly bind to the mRNA of ferritin and the transferrin receptor. Interestingly, this binding may be controlled by redox changes in the microenvironment. The iron responsive elements, which can be inserted into the 5' untranslated region of any gene and thereby make it possible to switch on the translation of that gene with iron, are of great potential importance as biotechnology tools, and have therefore been patented. In order to identify other genetic elements critical to iron metabolism, studies are being conducted on an iron dependent yeast, in particular on the mechanisms involved in transport of iron across cell membranes, a major gap in the understanding of human iron metabolism. These investigations focus on the genetics of an iron reductase, which appears to play a role in the initial events in membrane transport. Another group is studying the T cell antigen receptor, one of the most complex membrane receptors known, and has succeeded in cloning and sequencing yet another of the polypeptide chains of the receptor, the zeta chain, and in elucidating the function of another protein component, the eta chain. To determine their individual functions in the T cells, use has been made of mutants which do not synthesize these components. Although eta is present in only one tenth the amount of zeta, it has a central role in coupling receptor binding to intracellular activation. Preliminary data show that zeta and eta are structurally related; at present the precise genomic nature of this relationship is being examined. A further ongoing project concerns the post-translational assembly of receptor chains and their delivery to the cell surface. The group investigating that question previously discovered an extra-lysosomal degradative system within the endoplasmic reticulum, and has now gained some understanding of how this system distinguishes between species that are to be degraded and those that are not; it appears that unassembled subunits of the receptor complex are much more susceptible to degradation than the complete complex. Knowledge of the events occurring during post-translational transport of proteins to the cell surface has been applied to the study of such processes in the HIV-infected T cell, in collaboration with a group in NIAID. The precursor of the envelope glycoprotein of HIV is cleaved after it leaves the endoplasmic reticulum, and the cleavage products form the HIV envelope. It appears that the cleavage of the glycoprotein, which is very inefficient, is very sensitive to certain amines, which may have important clinical potential for the treatment of AIDS. The mechanism of T cell activation is another project vigorously pursued by the CBMB. When T cells are activated, intracellular protein kinases are stimulated. Their substrates are being identified and the regulatory consequences of their phosphorylation are being explored. Another aspect of T-cell activation is interleukin-2 dependent T cell proliferation. Interleukin-2, or T-cell growth factor, expresses itself via a receptor which another CBMB group has attempted to characterize over the past few years. Particular progress was made in purifying the ß-subunit of the receptor. By studying the genetics of unregulated expression in T cells infected with the HTLV-1 leukemia virus, this group has succeeded in identifying a binding protein which is involved in the control of transcription of the α-subunit of the interleukin-2 receptor. Of particular significance is the fact that this protein appears to be the same as, or closely related to, NF-kappa-B factor, which is involved in transcriptional control of a variety of other immunologically relevant systems, including immunoglobulin synthesis and the activation of the HIV virus. A further project of this group is to characterize other genes that are turned on when the T cell is activated. One such gene, called Act-II by the group, has been characterized. This gene encodes a small secreted protein which may be another lymphokine. Using a virus expression system in cultured insect cells, the protein has been isolated in large amounts, which will be very useful for more detailed biochemical analysis.

The Laboratory of Developmental and Molecular Immunity (LDMI) has been at the forefront of novel approaches to vaccine development. A major improvement was made in the vaccine against pertussis. As is well known, the currently available vaccine (DPT) has serious side-effects which, in many countries, have reduced its acceptance by the population, with a concomitant increase in the incidence of pertussis. After many years' work to find a safe, pyrogen-free and effective monovalent vaccine, a product (the toxoided pertussis toxin) was developed in this lab which, in preliminary clinical trials, has yielded very encouraging results. The trials have progressed to the stage of establishing effective doses. One of the most serious diseases for infants in this country is meningitis caused by Haemophilus influenza type b (HIb). The existing vaccine for children does not protect infants because it is a polysaccharide vaccine; polysaccharides do not activate T-cells, and are thus relatively poor antigens compared with proteins, especially in infants. This Laboratory pioneered another major advance in the technology of vaccine production by synthesizing conjugated antigens, consisting of a protective protein antigen which confers T-cell dependence (such as tetanus toxoid), chemically linked to the capsular polysaccharide. In the case of HIb, this approach has now come to fruition with the demonstration of protective antibody levels against HIb in infants during a preliminary clinical trial. The same principles have also been applied to various pneumococcus capsular polysaccharides, with the aim of protecting infants and children at high risk for pneumonia, such as those suffering from sickle cell anemia. Other pathogens under study are those that cause enteric fevers, which are a major public health concern in the developing countries. Attempts are being made to improve the immunogenicity of the LDMI's capsular polysaccharide vaccine against Salmonella typhi, which in its present form only confers 70% protection, by conjugating it with tetanus toxoid. The conjugation technology is also being used to produce new vaccines against Salmonella paratyphi and Shigella, and against an encapsulated pathogen which commonly causes bacteremia in hospital patients, Staphylococcus aureus. The LDMI also conducts basic research into developmental gene regulation in the immune system. As is now well known, the major histocompatibility antigen complex (MHC) is indispensible to T cell lymphocyte recognition of foreign antigens, although how precisely this is achieved is still enigmatic. Over the past several years considerable effort has been devoted by the LDMI to solving this problem. It is known that the expression of MHC genes is regulated by lymphokines, such as interferon and tumor necrosis factor, and these workers were among the first to describe regulatory sequences in the 5' flanking region of these genes. One, a highly conserved cis-acting sequence, is the interferon consensus sequence, and two proteins were detected which bind to this region after interferon treatment. This group's studies are also directed at the mechanism whereby MHC genes are turned on in the neonate. Another regulatory sequence exists in the 5' flanking region of the MHC genes, the so-called conserved class I regulatory element (CRE). Progress was made in determining the functions of three regulatory proteins which bind to three sites on the CRE. One such site appears to be of greater importance than the other two for regulation of transcription because the protein which binds to it is not detectable in fetal liver and brain, where MHC genes are not expressed; mutations in this region which impair binding are far more damaging to enhancer activity than those in the other two regions. The c-fos proto-oncogene is another gene which is induced at the neonatal stage and when cells of the immune system are stimulated with agents such as mitogens . Although c-fos does not appear to be involved in the control of MHC class I gene expression, the experiments showed an unexpected regulatory linkage between expression of c-fos and another proto-oncogene, c-myc. Another finding arising from the work on the induction of neonatal oncogene expression is that the neonate exhibits a unique transient protein factor which binds to the enhancer element of the c-fos gene. The characterization of these various nuclear binding proteins will be of great significance to the understanding of the function of these immunologically and developmentally important genes.

The Developmental Endocrinology Branch (DEB) continues its program of research into human disorders of growth and development. This year, the DEB has been particularly concerned with the control of reproduction, improvements in the diagnosis and treatment of Cushing's syndrome, and the control of linear growth. As part of its continuing study on the role of progesterone in controlling both ovulation and the function of the endometrium, the progesterone antagonist RU 486 continues to be an invaluable tool. Using this drug, it has been possible to strengthen the evidence that progesterone signals from the ovary are important in stimulating gonadotrophin release from the pituitary. Moreover, the drug is capable of delaying endometrial maturation in very low doses, establishing its potential as a contraceptive. In studies on the mechanisms regulating the normal reproductive cycle, investigators of the DEB have shown that the midcycle LH surge is due to changes in the amplitude, and not frequency, of pulsatile pituitary gonadotropin releasing hormone secretion, and that pulsatile gonadotropin secretion is largely abolished in hypogonadotropic hypogonadism and lactational amenorrhea. In studies of women with the so-called "Premenstrual Syndrome," no evidence was found that the syndrome has an endocrine basis; the main burden of these studies has thus been shifted to NIMH. Another group in the DEB continues to study the structure, as it relates to function, and the physiology of human chorionic gonadotrophin (hCG) and related glycoprotein hormones. By measuring hCG during the menstrual cycle with a sensitive, specific assay, early loss of pregnancy could be detected before women became "clinically pregnant". By this criterion about a third of all conceptions are lost. An unexpected finding is that early loss is more common among subsequently fertile than infertile women. Several radioimmunoassays were also developed to distinguish between various metabolites of hCG. Two metabolites in particular appear to have potential as cancer markers: the ß-core (constituting 99% of the immunoreactive hCG at certain stages of pregnancy), which was detected in male carcinoma patients, and a galactose-terminated metabolite which is only found in patients with gestational trophoblastic neoplasia. The DEB has several projects in progress concerning the physiology and pathology of the pituitary-adrenal system. The intractable hypercortisolism associated with the ectopic ACTH syndrome or adrenal carcinoma was successfully treated with RU 486, which has anti-glucocorticoid properties. RU 486 was also used to establish the manner in which glucocorticoids affect the immune response, evidence being obtained that these effects are glucocorticoid receptor-mediated. Using a clinical test based on the administration of ovine corticotropin releasing hormone (CRH), diagnostic refinements have been achieved to differentiate, on an outpatient basis, between Cushing's disease, ectopic ACTH syndrome and the hypercortisolism associated with depression. Also using CRH, it has been possible vastly to improve the treatment of pituitary Cushing's syndrome, which is caused by ACTH secreting adenomas. After CRH stimulation the adenoma is directly located by pinpointing the site of ACTH secretion; the surgeon can thus perform a ipsilateral hemipituitectomy, leaving no pituitary deficiencies. CRH is also helpful in differentiating between pituitary and hypothalamic causes of adrenal insufficiency. In vitro studies are yielding insights into the control of CRH release by the hypothalamus and placenta, and into the molecular biology of glucocorticoid receptors. Another focus of the Branch's activity continues to be human growth, its control and disorders. Preliminary results suggesting that many children with growth hormone deficiencies can be treated with growth hormone releasing hormone (GHRH) as an alternative to growth hormone per se, have been confirmed, which is of particular significance for children who develop antibodies to growth hormone. The accuracy of diagnosing growth hormone deficiency in prepubertal children by measuring spontaneous growth hormone release was compared with that of the classical stimulated release test, and it was found that the much more expensive spontaneous test identifies only half of those patients detected by the stimulated release test. Thus the stimulated test is still the preferable diagnostic tool, although there are limitations to its reliability, and therefore new approaches are called for. Human growth is also controlled by the sex steroids, and their role in promoting the growth of bone was studied in an animal model. Results strongly suggest that, among the sex steroids, the estrogens are of the greatest importance for growth. Significant advances were also made in the treatment of precocious puberty, especially in controlling the accelerated growth which accompanies this disorder. A pilot study demonstrated the usefulness of a combination of the antiandrogen spironolactone with the aromatase inhibitor testolactone in normalizing growth in gonadotropin independent sexual precocity. Testolactone was also very effective in the treatment of the ovarian cysts which occur in patients with the McCune Albright syndrome, a type of precocious puberty in females. One of the recent major clinical advances in the DEB has been the successful treatment of gonadotropin-dependent sexual precocity with LHRH analogues. In these long term studies, some children have now reached the average age of puberty, and their treatment was therefore discontinued; happily, it appears that their pubertal development is continuing normally.

The Laboratory of Comparative Ethology (LCE) is broadly concerned with the genetic, environmental and cultural regulation of behavioral development, and the stability and evolution of behavior patterns. Longitudinal studies concerned with the stability of behavior patterns in monkeys suggest that these may be partly inherited. This has been confirmed in pedigree analyses of individual differences in a variety of behavioral and physiological reactions to stress. Furthermore, field studies of wild troops of rhesus monkeys have underscored the generality and adaptive significance of stable individual differences in response to environmental challenge. A striking finding to emerge from these field studies is that inherited personality traits determine whether adolescent males become emigrant in the wild or not; the prevailing assumption had been that physical maturity or the mother's social dominance determined this behavior. Another focus of the LCE's investigations are the effects of environment on early human cognitive behavior and personality. For example, it was found that the more attentive and encouraging mothers are, the higher their infants score in noun comprehension and representational skills. The results of several studies have underscored the importance of attachments with significant adults in socioemotional development, and the impact of the quality of care received at home or from substitute care-givers on maturity and emergent social skills. Many new studies have been initiated this year, for which data collection are not yet complete. Among other questions, these will investigate the influence of maternal age, employment and parenthood status on infants' social and cognitive skills, the effects of emotional factors during pregnancy on infants' temperament and the mutual adaptation of parent and infant, and the nature of the effects of domestic violence on 10 to 12 year old children. The effects of cultural differences in care-giving traditions in Japan, Israel and North America on infants' later cognitive and social behavior are being investigated in a long term study. Another concern of the LCE is the origin of language, particularly in terms of its genetic, neuroanatomical and physiological correlates. The development of vocal interactions between infant monkeys, their mothers and aunts, and juvenile vocalizations during play, have been described in great detail. Projects with other New World monkeys have been concerned with the heritability of calling patterns and with the biochemical pathways responsible for vocal behavior. Physiological correlates of the isolation call production in adult squirrel monkeys have been found by establishing that the duration of call and pitch instability are correlated with plasma ACTH levels.

The Endocrinology and Reproduction Research Branch (ERRB) has continued its studies on the mechanisms of peptide hormone secretion, the hormonal regulation of cellular metabolism, and on the structure/function relationships of peptides and peptide hormones. Several groups in the ERRB have continued their investigation of the mechanisms of peptide hormone action, particularly that of gondatropin releasing hormone (GnRH) and angiotensin II (AII), and are sorting out the details of their multiple intracellular effects. This year they have shown how the biphasic LH response of pituitary cells to GnRH is controlled; the first rapid phase may be mediated by arachidonic acid, whereas protein kinase C (PKC) activation is probably responsible for the second sustained phase of release. Ca^{2+} responses to GnRH are also biphasic, with mobilization of intracellular Ca^{2+}, via inositol triphosphate, occurring in the first phase and an influx of extracellular Ca^{2+} during the second phase. Studies on the action of AII, which is coupled to a similar second messenger system, have revealed some new pathways for inositol phosphate catabolism and phosphorylation, and their relative significance is now under investigation. Another aspect of this type of signal transduction pathway to be investigated in great detail by ERRB workers is the role of the crucial component, PKC. How a single enzyme can control such diverse functions as growth, metabolism and signal transmission in the nervous system is still not known. A group of ERRB workers has discovered that PKC exists in different isozyme forms, encoded by separate genes. By studying the distribution of three isozymes of PKC, particularly in the brain, they hope to determine the function of the different isozymes. For example, isozyme I is found only in the brain and is localized in the dendrites of the Purkinje cells, whereas isozyme III occupies the cell body. In the neonatal brain, the appearance of type I is correlated with a burst in formation of nerve cell connections, suggesting that type I is involved in signal transmission. Turnover of PKC is an important part of this pathway, and it has been shown that PKC isozymes also have different susceptibilities to proteolysis by trypsin and endogenous proteases. Another approach to determining differences in the functions of various isozymes is to correlate secretory response of cultured cells with isozyme levels, and to determine their differing biochemical characteristics, studies which are now under way. Two more hormone receptors were purified this year, that of CRH and, by novel biochemical techniques, the very unstable AII receptor, which was further characterized by expressing target tissue mRNA in *Xenopus* oocytes. Further structural characterization of the LH/hCG receptors, which were purified last year, has shown them to be sialoglycoproteins. The other signal transduction system used by peptide hormones, such as LH, hCG and ACTH, is the so-called cAMP pathway. Many of the effects of these hormones can be explained in this framework, such as involvement of calmodulin in ACTH action, or the increased rate of phosphorylation of gonadotropin receptors from rat ovary and testis by c-AMP dependent protein kinase following receptor occupancy. Another consequence of gonadotropin action in the Leydig cell is thought to be stimulation of the P-450 cytochrome, which catalyzes P-450 dependent 17α hydroxylation of steroids, one of the critical steps in the production of testosterone. Evidence for this was obtained by finding increased levels of P-450$_{17\alpha}$ mRNA both *in vivo* and in cultured fetal Leydig cells, using a rat P-450$_{17\alpha}$ cDNA probe. A particularly interesting finding is that cultured fetal Leydig cells produce ß-endorphin under the influence of gonadotropins. From this and their earlier discovery that the Sertoli cell bears receptors for this opioid, and that FSH-stimulated androgen binding protein production is inhibited by opioids, one can conclude that the Leydig cell has a paracrine inhibitory function, which is probably of greatest importance before sexual maturation. Another group in the ERRB is interested in the question of how pregnenolone, the product of cholesterol side chain cleavage in the mitochondrion, moves out of this organelle. A pregnenolone-binding protein has been isolated, and at present its adrenal cortical distribution and structure are being studied. Another group in the ERRB is concerned primarily with the molecular structure of biologically active peptides. Workers in this group have synthesized several interesting peptides this year, for instance ^{10}B-derivatives of CRH and gonadotropin releasing hormone, without perturbing their biological activity. These boronylated derivatives are toxic to their target cells when irradiated with slow neutron gas, and have great potential both as therapeutic agents and in the study of heterogeneous cell populations. Another interesting group of newly synthesized compounds are analogs of the magainins. The natural magainins, which were discovered by NICHD investigators, are broad spectrum antimicrobials from frogs, with hemolytic activity. The group found that they could vary antimicrobial and hemolytic activity independently in synthetic analogs, resulting in a peptide with both high antimicrobial and low hemolytic activities.

The laboratory of the Deputy Scientific Director is concerned with the mechanism of action of nerve growth factor (NGF). It is a typical peptide growth factor, structurally related to certain oncogene products, and responsible for differentiation of certain types of nerve cells. An understanding of its mode of action will shed light on the mechanisms of differentiation in general. From studies conducted by this group it is now clear that NGF acts via the phosphatidylinositide pathway and causes a calcium dependent stimulation of arachidonic acid release. A biochemical consequence of NGF treatment appears to be stimulation of a specific ribosomal protein kinase, for which the ribosomal protein S_6 is the substrate. The mitogen, epidermal growth factor, also stimulates phosphorylation of S_6, but by a different kinase. This finding suggests that S_6 may play a pivotal role in determining whether the cell divides or differentiates. Many other phosphorylations occur after NGF treatment, and the search is on for the biochemical step connecting all these independent events. A finding of potential clinical and experimental importance is that glucocorticoids inhibit the expression of the neuron-glia recognition molecule, N-CAM, stimulated by NGF. Cells can now be obtained which lack this surface determinant, but are otherwise fully differentiated, which will help in the elucidation of this process.

In the laboratory of the Scientific Director, research is conducted with the goal of understanding how DNA damage is repaired in mammalian cells, and how unrepaired damage is converted into permanent mutations. This is fundamental to the elucidation of the causes of inherited diseases and abnormalities of cellular differentiation, such as tumorigenesis and birth defects. Work has continued on the biochemical repair mechanisms using a transfected UV-irradiated plasmid; the plasmid is not expressed if the UV damage is not repaired. It was found that pretreatment of cells with carcinogens enhances the cells' ability to repair DNA damage. Work is in progress to examine the cellular factors responsible for repair enhancement; a family of inducible proteins which bind specifically to damaged DNA has been identified. The genetic mechanism by which DNA lesions are converted into mutations is commonly known as mutation fixation. Work by this laboratory on mutation fixation has been greatly aided by the use of a so-called shuttle vector, pZ189, based on the SV40 virus. pZ189 has proved very useful in studying the direct and indirect effects of two typical DNA damaging agents, UV and a derivative of the carcinogen benzpyrene. The vector can be treated with mutagens (in vitro or in vivo); it is replicated in animal cells, harvested, and then amplified in bacteria to produce sufficient material to analyze the resulting mutations. Mutational specificity was demonstrated for these two DNA damaging agents. Treatment of cells with general stressing agents, such as phorbol esters, increases the frequency but not the nature of the mutations; the increase in mutational frequency is mediated by a secreted cellular factor. Several important conclusions can be drawn from this work. One, for example, is that general cellular stress enhances the propensity to convert transient DNA damage into permanent mutations, with important implications for the measurement of cumulative risk from carcinogens and other DNA damaging agents. Another is that, for the first time, a system has been shown to exist in mammalian cells which is analogous to the "SOS" pathway in bacteria. Finally, one group in this lab is focused on factors which positively or negatively control DNA replication in animal cells. These workers previously identified a mitogenic inhibitor (MI) in SV40-transformed hamster cells which is similar but not identical to TGF_{β}. This year, it was found that MI acts at a novel point in the cell cycle, the G1/S boundary, and its expression appears to be the inverse of EGF.

The Institute continues to acquire additional laboratory space. Construction of a three-floor addition to Building 6 has provided 25 new laboratory modules, accommodating the Laboratory of Developmental Pharmacology, one section of the Laboratory of Developmental and Molecular Immunity, and the DNA/protein Sequencing and Synthesis Unit of the Endocrinology and Reproduction Research Branch. During the past year, new space was renovated in Building 10 for the Laboratory of Theoretical and Physical Biology, allowing all of the LTPB's investigators to be housed in one location. Within the neuroscience complex (Buildings 36 and 37), we gained 25 additional modules for neurobiology research. This space is now being renovated and will be ready for occupancy early in 1989. Also during 1988, the Congress appropriated funds for still another addition to Building 6, now under construction. This wing ("Building 6B") will house one of the country's largest transgenic mouse facilities, as well as 10,000 square feet of additional laboratory space for the Laboratory of Molecular Genetics and a new department, the Laboratory of Mammalian Genes and Development. The animal facility will be ready in 1989, and the laboratory floors will be completed early in 1990. The Congress also appropriated additional funds for further work on a major new building ("Building 49") on the NIH campus that will allow continuing expansion of our neuroscience and neuroendocrinology research. This building will also house the neuroscience laboratories of two other Institutes as well as a state-of-the-art primate facility. Finally, NIMH transferred a small building to us ("Building 32"), adjoining the CBMB, which will allow further expansion of that Laboratory's research. We anticipate that the NICHD Intramural Program will have grown from 41,000 square feet of laboratory space (in 1983) to approximately 110,000 square feet by 1991.

In major personnel changes during the past year, Dr. Kathleen Dixon was granted tenure in the Section on Viruses and Cellular Biology; Dr. Mark Mayer in the Laboratory of Developmental Neurobiology; Dr. Shousun Szu in the Laboratory of Developmental and Molecular Immunity; and Dr. Lawrence Samelson in the Cell Biology and and Metabolism Branch. Dr. Michael Zasloff, Chief of the Human Genetics Branch, resigned this position to accept a position at the University of Pennsylvania School of Medicine, and he was replaced, as Acting Chief, by Dr. William Gahl. Dr. Richard Knazek resigned his position as Deputy Chief of the Developmental Endocrinology Branch to take a position in private industry, and Drs. Bruce Schrier and

Richard Sherins retired from their positions in the LDN and DEB, respectively.

Our clinical research fellowships for physicians being trained in investigative endocrinology or medical genetics continue to thrive, and in the past year, we developed new sources of support for postdoctoral (Ph.D. and M.D.) fellows who are receiving advanced training in basic science. These mechanisms include postdoctoral stipends paid by the Institute but which do not encumber regular government positions. These new fellowships are greatly enhancing our ability to offer postdoctoral training, especially for American graduates, and include an intramural National Research Scholarship Award (NRSA), a contractual program for the support of intramural training in biotechnology administered by the National Research Council of the National Science Foundation, and a new program that is intended for American postdoctoral candidates in any of the biological sciences (Ph.D. or M.D.). The latter mechanism (Intramural Research Training Award, IRTA) is the domestic equivalent of our long-standing Visiting Fellow program for postdoctoral candidates from abroad. Together with NIMH, we are also about to launch a new program for the support of intramural training in the neurosciences, to be administered by the National Research Council of the National Science Foundation. This neuroscience fellowship will accept its first applicants early in 1989. We have also been successful in identifying new donors of stipends, including endowments by private industry and foundations. NICHD funds administered by the NIH's Fogarty International Center have supported sabbatical visits by senior scientists from abroad under the terms of formal bilateral agreements between the NIH and institutions in Europe, Latin America, Asia, and the Middle East. Finally, a number of medical students supported by the Howard Hughes Foundation program at the NIH are working in this Institute during an elective year. Our summer student program was extremely successful during 1988, with more than 60 undergraduate, graduate, and medical students working in our laboratories.

Peer review of intramural research, conducted by the Institute's Board of Scientific Counselors and ad hoc experts, continues to receive great emphasis, with rigorous three-day site visits to each lab at four-year intervals (as well as a twice-yearly review of the Intramural Program in its entirety). During 1988, visits were made to the Laboratory of Comparative Ethology, Laboratory of Developmental Pharmacology, and the laboratories of the Office of the Scientific Director, with detailed critiques prepared as a result of these visits. The membership of the Board of Scientific Counselors reflects the increasing diversity of research interests within this intramural program, and currently includes Joseph G. Gall, Ph.D. (Chairman), Senior Member, Carnegie Institution (Baltimore); Stanley Cohen, Ph.D., Professor of Biochemistry, Vanderbilt University; Peter O. Kohler, M.D., President, Oregon Health Sciences University; Lynn T. Landmesser, Ph.D., Professor, Department of Physiology and Neurobiology, University of Connecticut; Lewis P. Lipsitt, Ph.D., Professor of Psychology, Brown University; Stanley G. Nathenson, M.D., Professor of Microbiology and Immunology, Albert Einstein College of Medicine; Allen H. Neims, M.D., Ph.D., Professor and Chairman, Department of Pharmacology and Therapeutics, University of Florida; and Savio L.C. Woo, Ph.D., Professor of Cell Biology and Molecular Genetics, Baylor College of Medicine.

During the past year we chose to merge our two neuroscience laboratories into one large neurobiology group, the Laboratory of Developmental Neurobiology, so as to promote greater synergism amongst our investigators and to conserve resources. This large Laboratory will be led by Dr. Philip Nelson. We have also created a new department, to be led by Dr. Heiner Westphal, the Laboratory of Mammalian Genes and Development (LMGD). This Laboratory will be concerned with many aspects of the developmental regulation of mammalian genes, with a particular focus on transgene biology. Dr. Westphal has recruited Dr. William Richardson from the Department of Biology, University College, London, as a second Senior Investigator in the LMGD.

Other developments in the past year include the continued strengthening of all aspects of the use of animals employed in our research, with our veterinary program directed by Dr. William Stokes. We have also continued to develop new computer-based administrative procedures in the Office of the Scientific Director so as to maximize the efficiency with which our resources are deployed. These new administrative approaches are ensuring the maximum yield with respect to scientific productivity while the current climate of constrained resources persists. A number of laboratories within the program are involved in various aspects of research on AIDS; they competed successfully for 1988 special set-aside funds for AIDS research above the Institute's normal budget ceiling, and it likely that this budget increment will be continued for the foreseeable future.

Seminars by the twelve Laboratories and Branches in this Program were numerous and well attended throughout the year, such that this Institute organized a relatively large fraction of the NIH's overall offering of intramural seminars and workshops. During the past year also, three major international conferences were organized by investigators in the Intramural Research Program, including: "The Second International Workshop on P450 Gene Regulation," hosted by the Laboratory of Developmental Pharmacology; "Gene Expression and Early Nervous System Development" (Laboratory of Developmental Neurobiology); and "Gene Regulation in the Developing Immune System" (Laboratory of Developmental and Molecular Immunity and the Office of the Scientific Director). During the past year also, the Institute hosted a major conference on "The Molecular Genetics of Development." This three-day conference was focused on the new information now available about how gene action controls embryonic development, and brought together leading molecular geneticists and developmental biologists from around the world who work in frog, fly, mouse and other animal systems. Speakers included Drs. John Gurdon, Frank Ruddle, David Hogness, Seymour Benzer, Gerald Edelman, Francois Jacob, Donald Brown, Joseph Gall, and Philip Leder, as well as a number of NICHD scientists. Other NICHD-sponsored conferences during the year included a meeting in Puerto Rico concerned with the ethology of free-ranging primates, organized by Dr. Stephen Suomi; a symposium on inborn errors of metabolism honoring the retirement of Dr. James Sidbury, the Institute's former Scientific Director; a meeting on the characteristics and consequences of maternal responsiveness, organized by Dr. Mark Bornstein; and a meeting in Beijing, China organized by Dr. Hao-Chia Chen and co-chaired by Dr. Arthur Levine, on the role of growth factors, oncogenes, receptors, and polypeptides in reproductive biology.

During 1988, the NICHD was especially honored by the choice of Dr. Igor Dawid (Chief, Laboratory of Molecular Genetics) to be the new Editor-in-Chief of the Proceedings of the National Academy of Sciences. Dr. Richard Klausner received the Outstanding Young Investigator Award of the American Federation of Clinical Research (AFCR), for detailing the biological mechanisms that regulate how cells take in and use iron. Dr. Klausner also won the Yale University Alumni Award for achievement in basic and applied research. In this same Laboratory, Dr. Allan Weissman received the AFCR Clinical Research Trainee Award, based on his work on the T cell antigen receptor. Dr. Daniel Nebert, Chief of the Laboratory of Developmental Pharmacology, was invited to deliver the Ernest A. Summer Lecture by the Oregon Health Sciences University, and Dr. John Robbins, Chief of the Laboratory of Developmental and Molecular Immunity, was invited by the Danish Medical Research Council to deliver the plenary lecture at a meeting in Copenhagen organized to celebrate the NIH's Centennial Anniversary. Dr. Michael Zasloff was the plenary lecturer at another meeting organized to celebrate the NIH Centennial, held at the Weizmann Institute in Israel. Zasloff was also awarded the PHS Meritorious Service Medal for his discovery of the magainins. Dr. Gordon Guroff received the NIH Director's Award for his work on nerve growth factor, as well as his scientific leadership, and Dr. Kuo-Ping Huang won the same award for his work on the mechanisms of signal transduction and metabolic regulation. Dr. Kevin Catt received the Presidential Rank Award, the highest ranking civilian award for federal government employees, for his career-long accomplishments in cellular and molecular endocrinology. Drs. William Gahl and Robert Weisberg won the PHS Superior Service Award for their achievements in research on inborn errors of metabolism and the mechanisms of genetic recombination, respectively. Dr. Mark Bornstein was elected a Fellow in the American Association for the Advancement of Science. Finally, Dr. Arthur Levine was invited to be the founding editor of a journal devoted to cellular and molecular biology, to be published by Harcourt, Brace, Jovanovich, and entitled, "The New Biologist".

Additionally, many of the Institute's senior investigators held honorary lectureships and visiting professorships during the year; other awards for research accomplishments were given to a number of our scientists by various universities and societies. Finally, during 1988 we sponsored a distinguished senior scientist in the Fogarty Scholars-in-Residence Program, Professor Elwood Jensen of the University of Chicago, who worked in our laboratories during his sabbatical.

Six years ago, this Institute embarked upon an ambitious program designed to improve the quality and quantity of its intramural scientific productivity, even as the rate of growth in research support declined. As one reflection of our success in this regard, NICHD intramural scientists this year published more than twice the number of peer reviewed original scientific reports, appearing in journals of stature, than had been the case prior to the beginning of this new direction. A rank order survey of the various NIH Institutes with respect to their yearly number of publications in nine of science's most prestigious journals, normalized to Institute budget, included NICHD among the top three. Moreover, more than half of our postdoctoral scientists are now fully supported by a stipend awarded from a non-NIH source--reflecting, we believe, the current quality and productivity of this intramural program.

Arthur S. Levine, M.D.
Scientific Director

Laboratory of Molecular Genetics

Igor Dawid, Ph.D., Chief

Left to right: J. Kennison, T. Sargent, I. Dawid, J. Levin, H. Westphal, M. Cashel

The best-known experiment in embryology may still be the discovery of the "organizer" by Spemann and Mangold in 1924. Intensive work in the area opened up by these classical observations was one aspect of the wide-ranging investigations carried out by Laboratory members during the past year. Spemann and Mangold found that the implantation of a piece of tissue from the dorsal lip of an embryo into the ventral region of another gastrula led to the development of a second dorsal axis in the host. Most of this second axis was formed from host, not graft, tissue; thus it was said to have been induced by the dorsal lip which was called the organizer, and the phenomenon was termed embryonic induction. The physical basis of induction was much studied in the following decades with inconclusive and often frustrating results. Eventually, many biologists came to regard studies on induction as futile, almost as suspect - but this was just a response to frustration, not to any doubt in the reality or importance of the basic phenomenon. In the past year the field of induction has received a major boost from several key experiments, leading quickly to a renewed interest and invigorating pace of research in many laboratories; researchers in the Laboratory of Molecular Genetics have participated in this rejuvenation of research on embryonic induction.

The recent chapter of the induction story derives from three major advances. One is the finding, about 20 years ago, that the earliest induction in the amphibian embryo concerns the specification of the mesoderm during cleavage and blastula stages. These results, achieved primarily by Nieuwkoop, helped divide the organizer induction phenomenon into simpler sub-effects: Mesoderm induction during blastula and neural induction during gastrula. Mesoderm induction proved more amenable to study. However, further progress was again slow in subsequent years, in part because the general state of biological research was not ready for the next step. This changed with the introduction of recombinant DNA and monoclonal antibody technologies and their application to questions of development. These methods allowed the generation of molecular markers for specific tissues in the early embryo, thereby making it possible to assay inductive processes by more objective and quantitative methods than before. The third important step was the very recent finding by J. Smith in England of an inducing factor secreted by the XTC cell line into the medium, providing a convenient source of inducer for a variety of experiments. Several laboratories quickly made the connection between the XTC factor and growth factors, leading to rapid progress in the field.

The group headed by **Igor Dawid**, with a major contribution by Frédéric Rosa and in collaboration with Anita Roberts and Michael Sporn of the NCI, has focused on mesoderm induction in Xenopus laevis. This group confirmed that a factor or factors in XTC medium induces morphological and biochemical differentiation toward mesodermal derivatives in explants (animal caps) that otherwise would become ectoderm. A relationship between the inducing principle in XTC medium and transforming growth factor ß2 (TGF-ß2) has been established by showing that mammalian TGF-ß2 induces mesoderm in animal cap explants and that this activity is blocked by antibody against TGF-ß2. TGF-ß1 is not active in this assay. Purification of the active factor or factors from XTC medium is underway.

To analyze the immediate molecular consequences of induction, several cDNA clones have been isolated that correspond to genes which are rapidly induced in animal caps by XTC factor; some of these clones also respond to fibroblast growth factor (FGF) which has been implicated in certain aspects of mesoderm induction. Study of these clones may help us to analyze signal responses in the embryo. Thus, molecular studies of embryonic induction are well underway and promise to illuminate this important and traditional area of biology in a new way.

Beyond the studies on mesoderm induction, researchers in this group have isolated a series of cDNA clones that are specifically expressed in the developing nervous system of Xenopus from gastrulation onward. One of these clones is a neurospecific ß-tubulin while the nature of the others is still unknown. These clones will be very useful in studies on neural induction which have been initiated.

Tom Sargent and his colleagues are interested in the control of gene expression in the early Xenopus embryo with particular emphasis on the question of how regional differentiation is established initially. It is clear that information localized in the egg is an important source of such specialization in addition to the role played by inductive interactions. The model system studied by this group concerns the activation of epidermal keratin genes, shown previously to be an early and cell-autonomous process. Sargent and collaborators have injected keratin gene constructs into fertilized eggs and regenerated the temporal and spatial controls of expression. The relevant control region has been localized in the 5' upstream sequences of the gene, and efforts to find protein factors involved in this regulation are underway. Such factors may make possible a molecular approach to the question of localization of developmental information in the egg, a classical problem of embryology.

Michael Cashel and his colleagues have continued their efforts to understand how a cell can coordinate the expression of its genes during balanced growth as well as during transient nutritional impoverishment. This group focused on the role played by the regulatory nucleotide, guanosine 3',5'-bispyrophosphate (ppGpp) in mediating these cellular responses in E. coli; ppGpp occupies a central position in the regulatory network of the bacterial cell.

A few years ago, Cashel and collaborators began characterizing the genes and the regulatory elements governing the metabolism of ppGpp. This information has been exploited to disentangle cause and effect relationships by artificially manipulating intracellular ppGpp levels; in normal circumstances nutritional conditions dictate ppGpp levels so that their effects cannot be separated. Previous reports have described the cloning and sequencing of the relA gene (which catalyses ribosome-dependent ppGpp synthesis during aminoacyl tRNA deprivation), the spoT gene (which catalyses ppGpp degradation to GDP), and a low level residual synthetic activity that persists despite deletion of the entire relA gene. In the past year this group has identified genes in the spoT operon that give a relA gene-dependent phenotype when interrupted as well as one that is a probable subunit of RNA polymerase, the omega subunit. Further, these workers have discovered cis-dominant complementation functions associated with mutations of these genes that suggest complex interactions at a regulatory or subunit association level. Most surprisingly, the source of residual ppGpp synthetic activity has been localized to the spoT operon. One of the requirements for this synthetic activity is the spoT gene product itself, which normally catalyzes ppGpp degradation.

Artificial induction of high levels of ppGpp under nutritionally sufficient conditions stops cellular growth and exerts regulatory effects on gene expression in a manner that is very similar to the responses seen when ppGpp is induced naturally during nutritional stress. Thus, stopping growth during starvation might not be due to nutritional deprivation per se but instead to a ppGpp-sensitive step that has protective value for the cell. The isolation of ppGpp-resistant mutants defective in this step is being pursued as a way to analyze the mechanisms involved in these phenomena.

Robert Crouch and his colleagues are interested in RNA processing, and specifically in the structure and function of ribonucleases H, a ubiquitous group of enzymes which degrade the RNA strand in a DNA/RNA duplex. These enzymes may be involved in RNA processing as well as in DNA recombination and replication. As an approach to structure/function studies, ribonucleases H from bacteria and retroviruses have been examined for enzymatic activity when either the amino or carboxyl termini were altered. Addition of residues at the amino terminus of Salmonella typhimurium RNase H had little effect on the specific activity of the protein, nor did a small deletion of the carboxyl terminus. In contrast, removal of the carboxyl one-third of the RNase H portion of the AKR MuLV reverse transcriptase RNase H dramatically decreased the RNase H activity without any significant alteration of the polymerase activity. Substitution of seleno-methionine for methionine in E. coli RNase H does not seem to alter the activity.

A second related project involves ribosomal RNA processing. Yeast is a favorable eukaryotic cell for such a study since both molecular and genetic tools may be applied. Crouch and his colleagues have isolated the RRP1 gene from yeast; mutations in this gene can result in abnormal processing of ribosomal RNA. The mRNA from the RRP1 gene has three unusual properties: 1) There is a long (for yeast) 3'-untranslated region; 2) the level of mRNA decreases as the cell density increases; and 3) there is an overlap of sequence of the 3'-terminus of the RRP1 mRNA with the 5'-terminus of a more abundant 0.6 kilobase mRNA. The RRP1 protein may be important in regulating the amount of pre-ribosomal RNA converted to mature rRNA.

Igor Dawid and collaborators have continued their studies on homeotic and other developmental genes in Drosophila with the analysis of the maternal locus fs(1)h and the regulatory locus trithorax (trx). The fs(1)h gene has been studied by sequence analysis of overlapping cDNAs corresponding to the major ovarian transcripts of 7.6 and 5.9 kb. The 5.9 kb mRNA sequence predicts a protein of approximately 110 kd, the 7.6 kb RNA a protein of 205 kd. The predicted proteins are very rich in glycine, alanine and serine, some of which occur as clusters. The proteins contain several potential asparagine-linked glycosylation sites and transmembrane domains. Antisera have been prepared against fusion proteins that contain portions of the predicted fs(1)h products; use of these sera supports the view that the fs(1)h products are membrane proteins. Staining patterns in progeny of fs(1)h mutant females show very early defects in the expression of products of the evenskipped, engrailed, and Ultrabithorax genes, suggesting a defect in initial segmental organization. The trithorax gene, a major regulatory developmental locus in Drosophila, encodes two large RNAs of 12 and 15 kb at all developmental stages tested, but the proportions of these RNAs vary. Fusion proteins derived from portions of these RNAs have been prepared and antibodies obtained. Their use promises insights into the nature of the trx protein products.

Proteins complexed with RNA in RNPs are believed to be important in RNA processing, transport, and turnover. Studies of functional properties of such proteins will be aided by genetic analysis, which is not possible in the mammalian systems employed most commonly for RNP protein work. It is therefore useful that Susan Haynes has isolated a gene encoding a Drosophila RNP protein; the gene has been sequenced and its chromosomal location determined. A related gene has been isolated by cross-hybridization; it encodes a distinct RNP protein, and is located on a different chromosome. These studies may open the approach to a genetic study of RNP protein genes in a favorable animal system.

The developmental genetics of Drosophila are also the focus of interest of James Kennison who joined the Laboratory recently. Kennison's interest is to expand our knowledge of loci that affect segment identity in this organism. In a novel approach to this subject, genes involved in the determination of segmental identity in D. melanogaster have been identified on the basis of dosage-dependent genetic interactions with genes already known to have a role in the process. Of eighteen genes identified by interacting mutations, twelve were not previously known to be involved in the process. Four of the newly-identified genes, the brahma, kismet, osa, and moira genes, were chosen for more extensive genetic and molecular analyses. Cells lacking either kismet or moira gene products in mosaic individuals survive and express homeotic transformations in some segments of the adult cuticle. These results imply that many of the new loci, selected by interaction with other loci, themselves have homeotic phenotypes. Both maternal and zygotic expressions of the brahma gene are required for survival of the early embryo. Lack of functional brahma products at either stage severely disrupts development. DNA from the region of the genome containing the brahma gene has been isolated and two candidates for the brahma transcriptional unit have been identified by insertional mutagenesis. Each of the two candidates for the putative brahma transcriptional unit encodes a single mRNA species present at all developmental stages examined. cDNA clones corresponding to both mRNA molecules have been isolated and sequenced. DNA from the chromosomal region containing the kismet gene also has been isolated. An insertion of P element DNA into the osa gene has been isolated and characterized in order to clone the wild-type osa gene. These studies promise to widen significantly the range of developmental genes that are available for study, and thus will be important in understanding the complex network of interactions that regulate embryogenesis.

The study of the regulation of gene expression by environmental or developmental cues is a key problem in modern biology. Much of this work has focused on control at the transcriptional level, but translational control is equally important in the cell's metabolism. Possibly the best-studied example of translational control in an eukaryotic cell is the regulation of GCN4 in yeast, the subject studied by **Alan Hinnebusch** and his colleagues. The GCN4 protein is a transcriptional activator of many enzymes concerned with amino acid biosynthesis. Through a complex cascade of regulatory loci the cell responds to nutritional conditions by adjusting its capacity for synthesis of amino acids; GCN4 is the proximal regulator in this cascade. While GCN4 mRNA synthesis is constitutive, synthesis of the respective protein is regulated. As Hinnebusch and his colleagues have shown, this regulation is mediated through sequences in the 5' untranslated region of the mRNA. The mechanism of this regulation has been studied further in the past year.

Translational control of GCN4 expression is mediated by AUG codons followed by short open reading frames in the 5' leader of the transcript: The third or fourth AUG codon is needed for repression in non-starvation conditions; the first is required for derepression in starvation conditions. Positive (GCN) and negative (GCD) trans-acting factors modulate the interactions between these upstream AUG codons. The following advances in our understanding of this translational control mechanism were made: (1) The regulatory functions of both the 5' proximal and 3' proximal AUG codons can be mimicked by heterologous upstream open-reading-frames (URFs), demonstrating a lack of strict sequence specificity for URF regulatory functions. However, placing URF1 downstream from URFs 3-4 abolishes regulation, showing that important sequence differences exist between these elements, and their 5'-3' order is critical. (2) Substitutions at URF1 with sequences found at URF4 show that the coding region and sequences 3' to the stop codon distinguish the functions of URFs 1 and 4. These results suggest that ribosomes must first translate URF1 and resume scanning to move beyond URFs 3-4 in derepressing conditions. (4) lacZ fusions to URFs 1, 3, and 4 are all efficiently translated when no upstream AUG codons are present. Moreover, URFs 1 and 2 have nearly the same weak inhibitory effect on translation of URF3-, URF4-, and GCN4-lacZ fusions, arguing against differential effects of URFs 1-2 on translation of URFs 3-4 versus GCN4.

GCN4 regulation depends on several GCN and GCD factors which act upstream in the regulatory cascade, between the nutritional state-sensing mechanism and GCN4. The following results were obtained: (1) Immunoblotting with antisera raised against GCN3 and GCD1 shows that these factors are expressed constitutively, supporting the notion that their functions are controlled by protein-protein interactions. (2) gcd12 and gcd2-1 mutations were shown to be alleles of the same gene that have allele-specific interactions with GCN3; the carboxyl-terminus of GCD2 was found to be homologous to GCN3. Based on these findings, it is likely that GCD2 contains two domains, one of which competes with GCN3. (3) Complementation mapping and DNA sequence analysis of GCN2 was completed; a mutation in a conserved lysine residue in the putative GCN2 protein kinase domain was shown to completely inactivate GCN2 positive regulatory function. (4) Mutations in the structural genes for two yeast eIF2 subunits (sui) behave like gcd mutations in causing derepressed GCN4 expression independent of the positive regulator GCN2. These results should help unravel the complex interactions of genes and gene products which result in the adaptive response by the yeast cell to changing nutritional environments.

The group led by **Judith Levin** aims to define the molecular mechanisms involved in the replication of mammalian retroviruses and in particular, to understand the factors which influence the regulation and expression of viral genetic information. Studies are being carried out with the murine leukemia virus system. Current interest is focused on the organization of the MuLV pol gene and on correlation of genetic structure with pol-associated enzymatic functions. Molecular clones containing MuLV reverse transcriptase sequences are being expressed in E. coli. The enzyme expressed by one of these clones, pRT250, was previously shown to have normal MuLV polymerase activity, but only barely detectable levels of RNase H activity. The deficit in RNase H activity has now been correlated with the absence of almost half the amino acid residues comprising a C-terminal region with homology to the E. coli and yeast RNases H. These results support the idea that the catalytic sites for polymerase and RNase H are localized to the N- and C-terminal portions of reverse transcriptase, respectively. Experiments to explore the functional relationship between these domains and to identify functionally significant sequences within the domains are in progress.

Retroviral mRNAs encode different proteins in adjoining units that are separated by translational stops or frameshifts. Special mechanisms must occur to allow expression of all of the open reading frames from these genomes. Such translational control of viral gene expression is being investigated in a separate project carried out by this group. Efforts are focused on the role of tRNA in readthrough and ribosomal frameshift suppression at retroviral gag-pol junctions. Suppression of the in-frame UAG termination codon separating the MuLV gag and pol coding regions has been demonstrated in an in vitro system. Yeast tyrosine amber suppressor tRNA as well as partially purified tRNA fractions, including glutamine tRNA, from MuLV-infected cells stimulate readthrough. Further purification of the mammalian tRNA species with suppressor activity is underway. In studies on ribosomal frameshift suppression, the distribution of isoacceptor tRNAs corresponding to amino acids present at or around the frameshift site is being analyzed. The results show that cells infected with HIV, HTLV-1, and bovine leukemia virus differ from control cells by a dramatic increase in the representation of some of these tRNAs in the hypomodified form. These modifications may be causally related to the observed frameshifting.

Robert Weisberg and his colleagues have continued their research into the mechanisms of genetic recombination and transcription of bacteriophages. They have shown that transcription of early genes in the λ-related temperate coliphage HK022 is broadly similar to that of its relatives, but nevertheless differs in an interesting way. HK022 expresses the first gene of its pL operon, which encodes Nun, a highly specific transcription termination factor, in the presence of prophage repressor. It appears to do this by synthesizing a novel repressor-activated transcript that begins immediately downstream of the pL promoter. Since no other pL operon genes are expressed in the presence of repressor, transcripts must terminate after *nun* in the presence of repressor but proceed through terminators in its absence. The diffusible factors assumed to be involved in antitermination of transcription have not yet been identified. If any are encoded by HK022, their genes are not located in the usual place for antitermination genes in other lambdoid phages.

HK022 and λ both encode proteins that promote recombination between special DNA sequences called attachment sites. The mechanism of site-specific recombination in the two phages is very similar, but the sites and one of the proteins (the Int protein) are not interchangeable. In order to localize the determinants that distinguish these two recombination systems, the primary structure of the HK022 attachment sites and recombination proteins were determined and compared to the analogous λ elements. Further, segments of the two phage attachment sites have been interchanged. This analysis shows that the critical determinants of the specificity difference are located in the central 50 bp (the core region) of the phage attachment site. Since Int protein binds to sequences within and outside of this segment, this finding suggests that the domains of Int that recognize the core region lie in the non-conserved regions of the two proteins, and that the domain(s) of Int that recognize the exterior regions lie in the conserved regions. These studies should provide detailed evidence on the structural requirements for Int protein function and thus lead to a better understanding of the mechanism of genetic recombination.

The application of the transgenic animal technique to fundamental questions in biology and to biotechnology has been among the most exciting biochemical research advances in recent years. **Heiner Westphal** and his colleagues have utilized these techniques in many imaginative ways. A major study is based on earlier work by these investigators which showed that a short region of DNA from the α-crystallin gene (the 5' regulatory region) could direct the expression of other genes to the lens. This knowledge was used to study the action of an oncogene (SV40 T antigen), a proto-oncogene (c-mos), and a DNA replication factor (polyoma virus large T antigen), respectively, in the lens of transgenic mice. Each of these factors affected cell growth in vivo in a distinct way: SV40 T antigen led to lens tumors, c-mos to a lens fiber differentiation defect, and polyoma T antigen to non-malignant hyperplasia. One outcome of these studies is that transgene-mediated cell transformation in vivo has now become a powerful way of immortalizing highly specialized cell systems such as the lens cell.

One aspect of the transgenic project addresses the human AIDS disease, and this work has produced an unsuspected finding. Of all cell systems in the transgenic mouse able to activate the HIV LTR, the Langerhans cell of the skin appears the most powerful. This enforces the view of strong macrophage involvement in AIDS pathogenesis. In another experiment of biomedical importance, transgenic animals were shown to be a potential source of human proteins of therapeutic value. Mice were generated that produce large amounts of tissue plasminogen activator (TPA) in their milk. This protein is used for the treatment of clotting disorders, (e.g., coronary artery occlusion), and its production in the milk of transgenic animals may be a practical way of obtaining large quantities of material.

In other transgenic studies, tissue-specific expression of murine P450 genes was analyzed (see report of the LDP). In situ analysis of expression of P_1 and P_3, two distinct P450 genes, has demonstrated the power of the transgenic technique in revealing the site of specific cell systems involved in the mammalian response to carcinogens and mutagens. The developmental role of homeobox-containing genes in mammalian embryogenesis was studied by analyzing the expression of two distinct homeobox genes in the developing embryo. As revealed by in situ hybridization, Hox 1.1 and Hox 1.5 are differentially expressed, and their expression coincides with specific events of pattern formation during midgestation. While the role of homeotic genes in Drosophila is well described, much less is certain about this role in mammals, and these experiments therefore hold considerable promise.

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* The bibliographies in this report include only those publications in which the first NIH author is an NICHD scientist. A more extensive bibliography, including collaborative reports and papers in press, is available on request. The staff members listed are those present in the Laboratory as of December, 1988.

Laboratory of Developmental Pharmacology

Daniel W. Nebert, M.D., Chief

The Laboratory of Developmental Pharmacology studies the molecular mechanisms of gene expression, mainly involving "P450" drug-metabolizing enzymes. The clinical discipline involving the study of genetic differences in drug metabolism has been termed pharmacogenetics. Cytochromes P450 are enzymes involved in the oxidative metabolism of steroids, fatty acids, prostaglandins, leukotrienes, biogenic amines, pheromones, and plant metabolites. These enzymes also metabolize innumerable drugs, chemical carcinogens and mutagens, chemicals in foodstuff, and other environmental contaminants. This large degree of overlapping substrate specificities, classes of inducing agents, and drug-drug interactions have caused great difficulty in P450 studies at the level of catalytic activities and protein immunochemistry, but at the level of metabolism, certain themes are clear. P450 enzymes, e.g., aryl hydrocarbon hydroxylase (Ahh), represent the classical "Phase I" metabolism in which the substrate is oxygenated. "Phase II" enzymes often use the oxygen as a site for further metabolism (e.g., quinone reduction, glucuronidation, and sulfate, glutathione, or glycine conjugation). Detoxification usually requires both Phase I and Phase II enzymes.

Hundreds of drugs and other chemicals are known to stimulate (induce) their own metabolism or the metabolic fate of structurally-related compounds. In addition, steroids, prostaglandins, and small peptide hormones have been found to regulate some of these activities. The mechanisms surrounding the induction of these enzymes and expression of these genes are of central importance to such fields as fundamental molecular genetics, developmental biology, teratogenesis, chemical carcinogenesis and mutagenesis, endocrinology, limology, and drug addiction, tolerance and toxicity.

The Section on Pharmacogenetics, under the direction of **Daniel W. Nebert**, is interested in the regulation and expression of genes encoding Phase I drug-metabolizing enzymes, most of which are P450-associated proteins, and certain Phase II drug-metabolizing enzymes. The P450 gene superfamily is presently known to comprise thirteen P450 gene families, eight of which exist in mammals. Several conclusions about P450 gene evolution are apparent. The P450 superfamily is ancient and has expanded via divergent evolution. The ancestral P450 gene, present probably more than two and a half billion years ago, had a minimum of 40 exons. Estimates of the unit evolutionary period (UEP; millions of years required for 1% divergence in amino acid sequence) range between 4 and 14, but are difficult due to several presumed instances of gene conversion between homologous P450 genes. Two mammalian mitochondrial P450 proteins, encoded by nuclear DNA, are more similar than the microsomal P450 proteins are to the prokaryotic P450 protein.

During the past year, striking differences in developmental-, sex- and tissue-specific P450 gene expression have been demonstrated by modern molecular biologic techniques. P450 expression vectors have also been used successfully to transform yeast and transfected into mammalian cell cultures. The Lab has extensively studied the CYP1A1 gene (trivial name, P$_1$450) in mouse hepatoma Hepa-1 cultures, and in aryl hydrocarbon receptor-defective and P$_1$450 metabolism-deficient mutant cell lines. These lines have been used as host cells for transfecting the reporter gene chloramphenicol acetyltransferase (CAT gene) driven by various lengths of CYP1A1 upstream sequences. It can thus be determined which upstream regions respond to a functional aromatic hydrocarbon (Ah) receptor and which regions specify P$_1$ metabolism. Upstream P$_1$450 regulatory sequences have been found to include: (i) the TATA box; (ii) a tetrachlorodibenzo-p-dioxin (TCDD)-inducible enhancer, which appears to include (iii) an element that augments constitutive gene expression; and (iv) a separate endogenous control element that may be involved in a negative autoregulatory loop. A homologous gene in the same subfamily, called CYP1A2 (trivial name P$_3$450), is under complicated control that is quite different from that for the CYP1A1 gene. Metabolism of substrate(s) by the product of the CYP1A1 gene not only controls its own constitutive expression but regulates the expression of genes encoding at least five other enzymes having coordinate metabolic functions--cytochrome P$_3$450 (CYP1A2), NAD(P)H: menadione oxidoreductase (NMO1), glutathione transferase (GT1), aldehyde dehydrogenase (AlDH1), and UDP glucuronosyltransferase (UGT1). All six genes have been cloned, are modulated by the aromatic hydrocarbon (Ah) receptor and induced by TCDD, and are defined as members of the [Ah] gene battery. Genes encoding the Ah receptor (the binding site for aromatic hydrocarbons), the putative repressor, and other trans-acting regulatory factors are being cloned and characterized.

Projects in this Section are divided among: (1) Basic molecular biology and genetics, (2) evolution of these genes and regulatory regions, including studies involving DNA sequencing, chromosomal walking and mapping, and (3) clinically important applications. Experimental systems include the use of recombinant DNA technology, inbred mouse strains, transgenic mice, and somatic cell genetics in culture. As an example of a clinically important application, the human CYP1A1 and CYP1A2 genes and flanking regions have been cloned and sequenced, and localized near the MPI gene on chromosome 15. Evidence has been accumulated to suggest that human CYP1A1 and CYP1A2 genes, similar to the orthologous genes in laboratory animals, are important in the activation of inert chemical procarcinogens, promutagens and proteratogens to active metabolites. Restriction fragment length polymorphisms (RFLPs) have been found, and families with high and low cancer incidence are being studied. In the future it should be possible to correlate RFLP patterns of these genes with the risk of human disease. Such tests would facilitate the evaluation of cancer and toxicity risk for individuals exposed to specific foreign chemicals. These assays would aid the individual, employer and physician in decisions regarding life style, cigarette smoking, employment, and prescription drugs.

The Unit on Microbiology, under the direction of Alvaro Puga, has been recently formed to coordinate efforts directed at the identification of the Ah receptor gene and other genes encoding trans-acting factors that affect CYP1A1 gene expression. Indirect evidence indicates that individual differences in the Ah receptor gene in human populations may be clinically relevant to an explanation of genetic susceptibility to environmental carcinogens and mutagens, as well as to drug-induced toxicity and birth defects. Projects in this Unit are divided among: (1) Basic and novel molecular biological approaches to cloning the Ah receptor gene, (2) development of expression vectors to study cytochrome P450 regulation and the role of aryl hydrocarbon hydroxylase in mutagenesis, and (3) identification of genes essential for development that are involved in detoxification processes.

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Laboratory of Developmental Neurobiology

Phillip G. Nelson, M.D., Ph.D., Chief

Left to right: J. Russell, P. Nelson, E. Neale, D. Brenneman, M. Mayer, D. Klein, A. Buonanno

This Laboratory is concerned with cellular and molecular mechanisms important for nervous system development. In pursuing this theme, the Section on Neurobiology, led by **Phillip Nelson**, has explored the concept of "Darwinian synapses", i.e., neural connections that survive during development because of their appropriate involvement in activity elicited by environmental stimuli. This concept has received considerable attention recently, and in continuation of work of some years' standing relevant to this notion, this Section (including Drs. Nelson and Yu in collaboration with Drs. Neale and Fields) has made significant progress recently in addressing the issue of differential synaptic development related to electrical activation of different populations of synapses. A three-chambered tissue culture system allows selective stimulation of one of two populations of sensory neurons from two side-chambers forming synaptic connections with ventral horn spinal cord neurons in the central chamber. Using intracellular recording techniques, this group can assess the number of axons innervating each spinal cord cell, and the size of the synaptic responses produced by these axons. Stimulation of a given set of sensory axons produces an increase in the numbers of axons maintaining synaptic connection by that set of axons and the unstimulated axons as well. Thus the number of axons connected is relatively unselectively increased by axonal activation. The strength of the synaptic connection is selectively increased, however, so that the stimulated afferents gain a competitive, 'Darwinian' advantage over their unstimulated counterparts. Consonant with earlier observations made in the LDN, these observations suggest that synaptic activity initiates two opposing processes that produce stabilizing, augmenting effects on the one hand and inhibitory or destabilizing effects on the other. Nelson et al. have begun the analysis of these two processes by manipulating the Ca^{++} ion concentrations in the bathing medium and selectively blocking an excitatory synaptic receptor, the N-methyl-D-aspartate (NMDA) receptor, during the period of chronic synaptic activation.

A major effort in the LDN during the past year has focused on investigating the neuronal deficits produced by the external envelope protein (gp120) of the human immunodeficiency virus (HIV). One of the confounding aspects of HIV is the diversity of strains and the problems this poses in efforts to neutralize this virus. The unit on Neurochemistry, led by **Douglas Brenneman**, has shown that the gp120s from three known strains of HIV, and two others which are yet to be characterized, all produce cell death in hippocampal cultures derived from fetal mice. These workers have shown that this cell death is mediated through the mouse homologue of the CD4 receptor. Interestingly, they found that antiserum made against an octapeptide of gp120 from the ARV isolate was able to prevent gp120-induced neuronal cell death caused by any of the HIV strains mentioned above. These data suggest some hope in counteracting the effects of the virus, despite its genetic diversity.

In addition to the gp120/neuronal cell death project, an effort was made in this Unit to examine peptide drugs that may prevent the gp120-mediated effects. Peptide T, an octapeptide sequence found in the external envelope protein, was found to antagonize potently and completely gp120-induced death in dissociated hippocampal test cultures. Analogs of the peptide T sequence found in other isolates of HIV were also shown to be active with this assay. Although the mechanism of action of Peptide T is not discernible from these early experiments, these studies indicate that this drug is effective in preventing neuronal cell death associated with gp120 and that it provides a rationale for peptide T to be tested as a therapy for the neuropsychiatric and neurological sequelae of AIDS.

The LDN has had a rich history in examining the physiological aspects of excitatory amino acids in the central nervous system. This year, another aspect of the NMDA receptor has been explored — its role in determining the structure and plasticity of developing neural networks. Brenneman and colleagues have found that NMDA antagonists can accelerate neuronal cell death during a critical period of development in cell culture. In addition, NMDA itself was found to increase the survival of neurons under conditions of electrical blockade. These data suggest that excitatory amino acids may have important "trophic" or developmental roles in addition to their recognized function as mediators of excitatory synaptic transmission.

The cell biology of neurotrophic interaction has been addressed by Ralph Alderson. He has identified two proteins of 15 and 40 Kd as the major substrate for vasoactive intestinal peptide (VIP)-induced phosphorylation in cortical astrocytes. He has shown an interaction between NGF and TPA in their effects on survival of cholinergic neurons in cultures from the medial forebrain of fetal mice.

The Molecular Neurobiology Unit, formally led by **Bruce Schrier**, now retired and succeeded by Andres Buonanno, has obtained four cDNA clones from differentiated mouse neuroblastoma cells and two from wounded rat cerebral cortex, the fusion proteins of which have neuronotrophic activity in one or more of a variety of assays. Sequencing of the inserts in these clones has not provided an understanding of the nature of their trophic activities. However, one of these clones, with exceptional amounts of trophic activity in the chick sympathetic ganglion assay, proved to contain a portion of the small subunit of mitochondrial ribosomal RNA. Experiments to determine the source of the neurotrophic activity of this molecule are in progress. Two cDNA clones from neuroblastoma mRNAs, when their transcripts are introduced into frog oocytes, cause the oocytes to be very sensitive to applied angiotensin II, resulting in the marked inward flow of negatively charged ions. Early sequencing data from one of these clones show the presence of intracisternal A particle sequences at both ends of the insert. The transcript of another clone from neuroblastoma cells causes oocytes to respond to the application of minute quantities of the head activator peptide (HAP), an undecamer which is known to be excreted by some neuroblastoma cells, perhaps in an autocrine regulatory scenario. The unit is pursuing this clone as a putative receptor for the head activator peptide. A cDNA clone from rat hypothalamus which bound one of two degenerate oligonucleotide probes which had been designed as the mRNA for HAP proved not to encode HAP. However, a clone from mouse neuroblastoma cells which also binds these oligomers is presently being pursued, as is the production of an antibody to HAP, a very poor immunogen. The group designed three oligonucleotide probes for conserved regions of the human alpha-2 adrenergic receptor for use in detecting the receptor from mouse tissues; they presently have one rat genomic clone which binds one of the oligomers and is being sequenced. Sequence data collected to date shows a >700 bp open reading frame and hydrophilicity regions which may be consistent with a receptor structure. The mRNAs of several tissues are being examined for the binding of the oligomers, for the purpose of obtaining a cDNA clone of the mRNA for the receptor. With the retirement of Dr. Schrier, some of these projects will be continued in collaborative arrangements. Dr. Buonanno has initiated studies directed at the molecular biological analysis of excitatory amino acid receptors and of axon-oligodendrocyte interactions.

Under the direction of **David C. Klein**, the Section on Neuroendocrinology has used the pineal gland to make fundamental advances in our understanding of signal transduction, both on a molecular-mechanistic basis and on a conceptual basis. In addition, the Section has made important advances towards a better understanding of the neural control of gene expression. The concept of biochemical "AND" gates has evolved within the Section during the past year. "AND" gates are transmembrane signalling mechanisms which integrate input from two sources. Like the electronic "AND" gates, which allow a signal to pass only if one input and a second are activated, biochemical "AND" gates control biochemical processes in a similar all or none manner. Two examples come from studies on pineal cyclic nucleotides, and a third comes from studies on the efflux of potassium ions from pinealocytes. Knowledge of the regulation of pineal cyclic nucleotides has expanded significantly within the last year as a result of intense and highly productive studies by Anthony Ho and Constance Chik.

Their efforts have allowed the Section to pioneer the analysis of dual receptor mechanisms which regulate cyclic AMP and cyclic GMP. Stimulation of pinealocytes by norepinephrine, the physiological transmitter of the pineal gland, causes 100- to 200- fold increases in both cyclic AMP and cyclic GMP. Norepinephrine is known to act through both α_1- and β-adrenergic receptors. Full stimulation requires activation of cyclases and of protein kinase C. Activation of adenylyl and guanylyl cyclase occurs as a result of receptor occupancy by β-adrenergic agonists or by VIP, or by postreceptor actions of cholera toxin or forskolin. However, these actions produce less than a 7% of maximal cyclic AMP response and less than a 3% of maximal cyclic GMP response. Full stimulation occurs only if protein kinase C is activated, which occurs as a result of α_1-adrenergic stimulation. Studies in the Section have indicated that α_1-adrenergic agonists act primarily through an elevation of $[Ca^{2+}]i$, which alone seems to cause translocation of protein kinase C and which is sufficient to cause full stimulation of cyclic AMP. In the case of cyclic GMP, the elevation of $[Ca^{2+}]i$ has a second effect, one which probably involves arachidonic acid.

The third example of an "AND" gate comes from studies on K^+ efflux, which were performed by Valentine Cena and David Klein. Several years ago Klein and his coworker Joan Weller discovered that norepinephrine produces a marked increase in the release of ^{42}K. Klein has continued to study this with Cena, and during the past year has discovered that this release seems to require the interaction of both cyclic AMP and of Ca^{2+}. As discussed above, the increase in cyclic AMP requires activation of both α_1- and β-adrenergic receptors. Release of K^+, however, does not occur if cells are loaded with cyclic AMP. Elevation of $[Ca^{2+}]i$ is also required. Thus there appears to be an "AND" gate which is regulated by both Ca^{2+} and cyclic AMP. Extensive studies on the identity of the gate point to the likely possibility that it belongs to a class of K^+ channels which are activated by Ca^{2+} and inhibited by charybdotoxin. These studies involve biochemical and patch clamp analysis. The biochemical studies provide the main support for the contention that Ca^{2+} alone is not sufficient to open this channel, but that cyclic AMP also seems necessary.

These studies provide clear models of biochemical "AND" gates. It seems probable that such "AND" gates function throughout the body to integrate input. They could play a critical role in the brain, both to decrease noise and to increase the selectivity of neural interactions, by requiring simultaneous activation of two membrane transduction systems. An outgrowth of the "AND" gate concept is the notion of multiheaded missiles--modern day versions of the elusive magic bullet. Multiheaded missiles would contain agonists which are carefully selected on their ability to operate "AND" gates in specific cells. The agonists chosen would not necessarily represent the physiological regulators of the cell, but would be able to act in a similar manner. For example, in the case of the pineal gland, the α_1- and β-adrenergic stimulatory action of norepinephrine could be mimicked by VIP and phenylephrine.

The Section on Neuroendocrinology has also made several important advances toward its goal of understanding how the enzymes in the tryptophan->melatonin pathway are regulated during development and by neural mechanisms. Major advances have been made in studies on tryptophan hydroxylase and hydroxyindole-O-methyltransferase. Joan Weller has isolated several sheep tryptophan hydroxylase DNA clones. Initial studies have indicated that at night, the tissue content of one species of tryptophan hydroxylase mRNA increases significantly. Studies performed using a human pineal cDNA library, in collaboration with Ed Ginns (NIMH), are leading toward the isolation of human tryptophan hydroxylase. Helena Illnerova, working with Joan Weller, has isolated several human pineal hydroxyindole-O-methyltransferase cDNA clones which are being characterized. The human pineal cDNAs will be used in collaboration with other groups for genetic analysis.

The Unit on Cell Biology, directed by **Elaine Neale**, has focused on the observation that tetanus toxin alters neuromuscular activity, but does not have a direct physiologic effect at the neuromuscular junction. It acts only after uptake at the nerve ending and retrograde axonal transport to the motor neuron cell body. Tetanus toxin receptors are believed to be gangliosides, although there is indirect evidence that a protein receptor may exist also. Investigators in this Laboratory have been studying various aspects of tetanus toxin-neuron interactions, in neuronal cell cultures, for a number of years. Dr. Neale has undertaken studies aimed, ultimately, at defining certain aspects of the cell biology of tetanus toxin action. These studies involve following the pathway of endocytosis of the toxin molecule and determining whether the organelles involved are different from those implicated in protein receptor-mediated endocytosis. Spinal cord neurons, in culture, exhibit a predictable electrophysiologic response upon exposure to the toxin. Intracellular localization of the toxin at selected times during the course of toxin action might suggest which organelles are involved in the production of paroxysmal depolarizing events, and which in the long-lasting electrical quiescence which ensues. Additional studies of toxin effects on neurotransmitter release, and on the survival of developing neurons, are planned.

Experiments to date have indicated that immunocytochemistry may provide a viable approach to intracellular toxin localization. A number of monoclonal antibodies are available, both neutralizing and non-neutralizing, and specific for known regions of the toxin molecule. Pre-mixing certain of these antibodies with toxin increases the levels of both toxin and antibody bound to neurons. The complex appears to be internalized, and disappears with a half-life that is similar to that of the toxin alone. Additional studies show that one particular antibody, which is non-neutralizing and specific for the Fragment C (binding) portion of the molecule, forms a complex with Fragment C which binds to the neuron surface, is non-toxic, and can be used as a substrate for immunofluorescence of living cultures. This probe can be used on neurons within two hours of plating, and appears to persist for several days. Studies are in progress to define the feasibility of labeling freshly dissociated neurons in suspension (prior to plating) such that they may be identified at some later time in co-culture with unlabeled neurons.

The Unit on Neuronal Secretory Systems, directed by **James Russell**, studies the nerve terminal, which is a highly specialized region of a neuron, separated from the neuronal soma by an axon, whose function is to release neurotransmitter quanta and to regulate the number of quanta secreted. Secretion of neurotransmitters and other biologically active substances from nerve terminals forms the fundamental means by which the central nervous system (CNS) operates from the time of development to higher order functions in the adult. Modulation of the quantity of the transmitter released at the terminal may form the basis for all central nervous system functions, including integration of information, as well as long term information storage and retrieval. This modulation is achieved by transduction of information content in the action potential train, and by local influences at the nerve terminal via activation of receptors at the terminal and the resultant modification of the responses of the terminal membrane. Because of their complexity (cellular heterogeneity and complex organization) and extremely small size, basic understanding of the molecular mechanisms of nerve terminal function in the central nervous system is lacking. The program of this group is focused on studying the biochemistry and physiology of the nerve terminal using the neurohypophysial neuroendocrine cells as the model system. The nerve terminals of the neurons of the hypothalamo-neurohypophysial system, which secrete vasopressin or oxytocin, are discretely localized in the neurohypophysis, where they are accessible to experimental manipulations both in vivo and in vitro. These nerve terminals can be isolated from the neurohypophyses without contamination by the post-synaptic membrane, unlike nerve terminals from other regions in the central nervous system.

Studies on the elucidation of the functional organization of the nerve terminal forms the central theme of this Unit. The current focus of the group is on the investigation of the importance of ionic channels and receptors on the initiation and modulation of secretion at the nerve terminal. Carolyn Bondy's experiments have shown that a type of K^+ channel may play a central role in modulation of secretion at the terminal caused by frequency information in the action potential train. Bondy also showed that this K^+ channel is blocked by dendrotoxin, a polypeptide toxin isolated from the venom of the South African green mamba, Dendoaspis angustyiceps. Kappa opiate receptors present on the oxytocin nerve terminals were shown to be involved in the modulation of oxytocin secretion. In these experiments, Bondy showed that dynorphin, cosecreted with vasopressin, specifically was involved in down-regulation of oxytocin secretion from the neighboring oxytocin terminals. The molecular mechanism by which kappa receptor occupation results in inhibition of oxytocin secretion is currently under investigation.

The neurosecretosome preparation (isolated neuroendocrine nerve endings) has been maintained in culture for long periods of time. These cultured nerve endings are being used to study the dynamics of hormone secretion, its modulation by receptor occupation, and for the identification of ionic channels on nerve terminals, using state-of-the-art biophysical techniques so that the channels, and their modulation by neuropeptide receptors on the nerve terminals, can be investigated. In collaboration with Elis Stanley, patch-clamp techniques are being used to characterize both the Ca^{++} and K^+ channel types present on the nerve terminals.

The neurosecretosome preparation has facilitated the study of the kinetics of secretion in response to depolarizing stimuli with very high temporal resolution. These studies revealed that during prolonged depolarization, secretion undergoes inactivation even when membrane potential is held at depolarizing levels. Kemal Payza has found that this inactivation is dependent on extracellular calcium ions and not caused by the membrane potential change alone. Membrane permeable analogues of cyclic GMP markedly alter this rate of inactivation. He has also shown that the inactivation process is highly temperature-dependent, suggesting an enzymatic process. He has used the nerve terminal preparation to identify intracellular second messengers involved in the modulation of secretion. The effects of both kappa opiate receptors and FMRF-NH_2 receptors on secretion, and their intracellular coupling, are being investigated.

The neurosecretosome preparation provides an ideal model to study intracellular reactions involved in triggering and regulation of neurosecretion. The use of toxins that block secretion has been shown to provide a means of identifying cellular substrates important in the exocytosis machinery. Holly Trenchard has shown that tetanus toxin at nM concentrations completely blocks depolarization-induced secretion from isolated nerve terminals. This inhibition is dependent on toxin internalization, and is blocked by tetanus antitoxin. Dr. Trenchard is attempting to reverse the inhibition of secretion by replacement of cellular metabolites to gain insight into the possible cellular locus of action of this toxin.

The high resolution video imaging microscope adds a new dimension in the investigation of the functional organization of the nerve terminal . Preliminary studies indicate that this instrument will be valuable in resolving long standing questions on the kinetics of Ca^{++} concentration increase in the terminal and its homeostasis. Furthermore, it is envisaged as a method to visualize and quantitate intracellular biochemical reactions with high temporal and spatial resolutions.

During the past year, an explosive increase in research on excitatory amino acids was noticeable at major scientific meetings, reflecting the now widespread realization that L-glutamate and perhaps related amino acids act as both fast excitatory neurotransmitters, and as modulators of processes as diverse as memory formation, neuronal cell death, and motor pattern generation. An understanding of the physiology, biophysics and cell biology of excitatory amino acid receptors is fundamental to experiments designed to probe these complex processes, and work in the Unit on Neurophysiology and Biophysics, led by **Mark Mayer**, is centered on studies of excitatory amino acid receptors in mammalian nerve cells grown in culture. Substantial progress was made in developing a fast perfusion system for rapidly applying excitatory amino acids and antagonists, and this is allowing experiments that were previously impossible for technical reasons, such as brief applications of saturating concentrations of agonists required for constructing dose response curves, as well as measurement of the rate constants of desensitization and of the binding and dissociation of antagonists.

The activity of the NMDA subtype of L-glutamate receptor is modulated by physio- logically important divalent cations, including magnesium and zinc.

Since zinc is present in excitatory nerve terminals, and released into the extracellular space, studies on its physiological action are of great interest. In cultures of hippocampus, Mark Mayer and Ladislav Vyklicky have found zinc to have two major effects on excitability, which are produced via several distinct cellular mechanisms. An increase in excitability reflects reduction of inhibitory postsynaptic potentials, by 50 μM zinc, coupled with a lowering of the threshold for action potential initiation, reduction of the spike after hyperpolarization, and block of spike accommodation during prolonged excitatory stimuli. Under voltage clamp, zinc suppresses a transient outward current, and antagonizes responses to the inhibitory amino acid GABA. Zinc also modulates excitatory synaptic transmission, and selectively reduces the slow component of epsps, with no action on the early synaptic response. Responses to NMDA are strongly antagonized by 50 μM zinc, while responses to kainate and quisqualate are slightly potentiated. Zinc antagonism of NMDA receptor responses is only weakly sensitive to the membrane potential, is not strongly sensitive to changes in the concentration of allosteric modulators such as glycine, and is reduced on raising the extracellular calcium concentration, suggesting that zinc binds to a unique site on the NMDA receptor complex.

Mayer and Vyklicky have used fast perfusion of responses to excitatory amino acids to reveal three patterns of response: Sustained activation of kainic acid receptors by kainic and domoic acids, slow desensitization of NMDA receptors (time constant 200 ms at 100 μM NMDA or L-aspartate), and very fast desensitization of quisqualate receptors by quisqualate, AMPA and L-glutamate. These kinetically distinct processes should be helpful in determining the pharmacological specificity of agonists acting at non-NMDA receptors, since differentiation between kainate and quisqualate receptors has been difficult prior to this. The lectin concanavalin-A selectively reduces desensitization at quisqualate receptors, but does not alter responses to kainate or NMDA, suggesting that the quisqualate receptor may be a glycoprotein.

Ian Forsythe and John Clements have continued to analyze excitatory synaptic transmission in cultures of hippocampus, and have discovered a presynaptic inhibitory action of L-glutamate on transmitter release, which appears to reflect the action of L-glutamate at a fourth type of receptor classified by activation using the synthetic ligand AP4. Discovery of the inhibitory action of L-glutamate on transmitter release is a major step forwards, and helps to assign a function to the AP4 receptor, for which no known function had been discovered. The very high potency of L-glutamate at the AP4 receptor suggests a significant role as an autoreceptor, regulating excitability via a presynaptic mechanism.

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Laboratory of Neurochemistry and Neuroimmunology

Yoke Peng Loh Ph.D., Acting Chief

This Laboratory is concerned with the development, functional organization and interactions between two major integrative systems in the body - the central nervous system and the endocrine system. The approach of the Laboratory is cell biological in nature, and hence utilizes a wide variety of techniques and concepts from a number of disciplines, e.g., physiology, biochemistry, morphology, immunology, and molecular biology. In particular, the lab studies various neuropeptides, intracellular membrane systems and receptors which are found in these organ systems and which are essential to their functions (e.g., regulation of neuropeptide gene expression, neuropeptide processing, neuronal morphology and function). A special emphasis is placed on the study of the cellular development of these systems and the role of precociously expressed neuropeptides on embryogenesis and neurogenesis.

The research goal of this Laboratory is to study brain and pituitary peptides which are involved in intercellular neurocommunication and neural development. The emphasis has been on the pro-opiomelanocortin (POMC, ACTH/endorphin/α-MSH) family of peptides and more recently the enkephalins. Endorphin and enkephalins are opiate peptides found in brain and pituitary; these peptides have been shown to have effects on nervous system development. α-MSH is present in brain and pituitary, but in humans it is present in the pituitary only during pregnancy and in the fetus. This peptide has been implicated to play a role in osmoregulation, fetal growth, neuronal differentiation and neurite regeneration. ACTH is a pituitary peptide which stimulates steroidogenesis and is a mediator of stress. Recently, another member of the POMC family (N-POMC 1-49) has been shown to be a potent mitogen for adrenal cells. All of these peptides exhibit various central nervous system effects and are thought to act as neurotransmitters and neuromodulators. The major focus has been to continue to study the enzymology and regulation of biosynthesis, packaging and secretion of the POMC family of peptides. In addition, the developmental expression of the POMC and enkephalin genes, as well as the anatomical distribution and role of these peptides in the developing and adult nervous system, were investigated.

The ACTH, α-MSH and endorphin peptides are synthesized in the intermediate lobe of the pituitary from a common, glycoprotein prohormone (pro-opiomelanocortin, POMC) of about 32,000 daltons in size. This group has assayed for several enzymes involved in the processing of this prohormone. These include a carboxypeptidase B-like enzyme, an aminopeptidase B-like enzyme, and a paired basic residue-specific prohormone converting enzyme (PCE). This latter enzyme has been purified to apparent homogeneity from secretory vesicles of the bovine pituitary intermediate lobe and neural lobe. PCEs from both lobes appear to have very similar characteristics and are likely to be the same enzyme. PCE is a glycoprotein, has a molecular weight of ~70,000 daltons, and cleaves several precursors (POMC, pro-vasopressin, pro-insulin, and pro-enkephalin) at paired basic residues to yield products seen in the tissues that synthesize these prohormones or neuropeptide precursors. Inhibitor studies have shown that PCE is inhibited by two aspartyl protease inhibitors, pepstatin A and diazoacetyl-norleucine methyl ester, but not by thiol or serine protease inhibitors. This finding identifies PCE as an aspartyl protease. Recently, Nigel Birch has screened a number of aspartyl protease antibodies by Western gel analysis and identified one anti-cathepsin D antiserum which recognizes both Cathepsin D and PCE, indicating a structural relationship between the prohormone processing enzyme and Cathepsin D. Based on this finding, Birch, in collaboration with M. Brownstein (NIMH), has designed a series of oligonucleotide probes to clone PCE. The probes are to specific regions of aspartyl proteases, some of which are conserved across all members of the family and others specific for one particular enzyme. An AtT-20 (anterior pituitary corticotroph) Okayama-Berg cDNA library has been screened at various stringencies by colony hybridization and clone blotting techniques. Two cDNAs which have strong similarity to human Cathepsin D have been sequenced. The identity of these clones suggests that there are at least two genes expressing Cathepsin-D-like enzymes, a hithertofore unreported observation. This is an exciting finding and raises questions concerning the subcellular distribution (besides the lysosomes) and function of these two Cathepsin D enzymes in the cell. Other cDNA clones which appear to have less similarity to "authentic" Cathepsin D are currently under investigation; one of these may code for PCE.

Further characterization of the cleavage specificity of PCE by Fernando Estivariz showed that the enzyme, in addition to cleaving POMC to yield ACTH, β-endorphin and a 16K glycopeptide, was also able to cleave N-POMC 1-76 (a fragment of 16K glycopeptide) at an Arg-Lys pair to yield N-POMC 1-49, the mitogenic peptide, and Lys-γ_3 MSH, both of which are found in the pituitary. The products formed were small enough to be accessible for identification by HPLC, immunological cross-reactivity with specific antibodies, and amino acid composition, showing unequivocally that PCE cleaved between the Arg-Lys pair of the substrate. Estivariz also showed, using the secretory vesicle membrane-associated form of PCE, that the enzyme was inhibited by EGTA, a strong chelator of Ca^{++}, indicating for the first time that PCE is a metalloprotease. This is an important finding since it raises the possibility that Ca^{++} may regulate the biosynthesis of POMC-peptides at the post-translational level.

Previous studies by Stela Elkabes have shown that during salt-loading stress, plasma ACTH and POMC mRNA levels in the anterior pituitary of mice are increased. To determine if two secretogogues of ACTH, CRF and AVP, are involved in mediating this response, Howard Tracer and Maria Castro have studied the mRNA levels and secretion of these two peptides in vivo during salt-loading, as well as the interaction of CRF and AVP at the cellular level in mediating ACTH secretion and regulation of pituitary POMC MRNA levels. Tracer, using a quantitative in situ hybridization technique, showed that AVP, but not CRF, mRNA in the hypothalamic neurons was increased after two days of salt-loading. Furthermore, immunoreactive CRF in the median eminence (the release site) was unaltered, but plasma AVP was increased. These results suggest that AVP may play a key role in potentiating ACTH secretion and POMC synthesis in the anterior pituitary during hypertonic stress. This is similar to other stresses, e.g., electroshock treatment and hypoglycemia, in which no change in portal or peripheral blood CRF is observed, but plasma AVP is increased significantly. Why in certain types of stress, ACTH secretion is potentiated by increased AVP levels in the presence of basal CRF levels, but in others (e.g., cold, heat-shock stress) by an increase in CRF, is an interesting question. Castro has used mouse dissociated anterior pituitary cells in culture to study the interaction of AVP and CRF on ACTH secretion to understand this issue. She showed that AVP at $\geq 10^{-8}$M in the presence of basal (10^{-10}M) CRF produced an ACTH secretion level equal to 10^{-9}M CRF. Prolonged treatment of the cells at 10^{-9}M CRF resulted in desensitization of the receptors and decreased ACTH secretion. Such desensitization was less evident when cells were treated with basal CRF and increased AVP. These findings indicate efficiency in the proposed mechanism for regulating ACTH secretion during salt-loading, i.e., AVP is the primary regulator of ACTH secretion with CRF playing a permissive role. Such a mechanism may be operative perhaps in chronic stress, whereas an increase in CRF may occur in acute stress paradigms.

Castro also analyzed the second messengers involved in mediating the CRF and AVP effect. She showed that CRF acts through a cAMP/protein kinase A dependant pathway, while AVP acts through a cAMP independent pathway involving phospholipase C, phosphoinositide turnover and protein kinase C. She found that unlike the situation in the rat, when AVP was added with CRF, the potentiation effect on ACTH secretion was not due to an enhancement of intracellular cAMP levels but to other mechanisms, perhaps involving the enhancement of phosphorylation of proteins mediating secretion. Dr. Castro has also demonstrated that AVP and CRF enhance POMC mRNA levels in anterior pituitary cells. This result suggests that these two peptides (CRF and AVP), in addition to having effects on ACTH secretion, play a role in upregulating POMC mRNA levels in the anterior pituitary during salt-loading stress.

Recently, a new project has been initiated to study the developmental regulation of expression of [met]enkephalin and POMC genes, and the role that peptides derived from these genes may play in neurogenesis and embryogenesis. Two model systems, the frog (Xenopus laevis) and the mouse, were used. Initial studies focussed on defining the temporal/spatial distribution of POMC and [met]enkephalin peptides during development. Stela Elkabes showed, using in situ hybridization histochemistry, that POMC mRNA appears very early in development, at embryonic day 10-1/2 (E 10-1/2) in the presumptive arcuate nucleus of the CNS. POMC mRNA did not appear in the pituitary until E 12-1/2 in the anterior lobe and E 14-1/2 in the intermediate lobe. Immunocytochemical studies indicate that the POMC mRNA is translated at E 10-1/2. The POMC neurons appear to mature very rapidly. Neurite outgrowth, arborizations and growth cones are evident at this early stage of CNS development. The POMC system appears to be expressed before other peptidergic systems studied in parallel (LHRH, oxytocin and vasopressin), suggesting an important role of POMC derived peptides in early neurogenesis. Studies by William Hayes have shown that POMC [met]enkephalin and TRH mRNAs appear during early development of the frog CNS, at stage 45 tadpoles. In addition, POMC mRNA could be detected as early as stage 33 embryos. This result again indicates the very early expression of the POMC system, as in mammals. Hayes has also mapped the anatomical location of the neurons expressing these mRNAs, both in adult and in the tadpole brain. He has recently begun studies to test the effect of naltrexone, an opiate receptor antagonist, on brain development in early tadpoles. May Wong has cloned the X. laevis [met]enkephalin gene and is currently mapping the 5' upstream regulatory regions, with the ultimate aim of defining the trans-acting factors which regulate the gene. Expression of these factors may be the key to triggering the tissue-specific expression of neuropeptide genes during development. Overall, these studies will lead to a better understanding of the timing and functional interplay of gene, peptide and receptor expression during development, and hence the developmental role of neuropeptides.

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Laboratory of Theoretical and Physical Biology

David Rodbard, M.D., Chief

Standing: P. Munson, A. Yergey, D. Rodbard; sitting A. Chrambach

This Laboratory has a unique program involving the application of several theoretical and physical methodologies to current problems in clinical investigation and fundamental research. The Section on Theoretical Biology, directed by **David Rodbard,** has continued its studies of mathematical modelling, statistical analysis, development of computer programs for biomedical research, and a combined theoretical and experimental approach to the characterization of complex systems of receptors for drugs, hormones and neurotransmitters. **Peter Munson** and his colleagues have given further emphasis to the development of general, flexible methods to describe families of curves, as arise in the context of dose-response curves, calibration curves, and when combining results from multiple experiments, subjects, or treatments. The FLEXIFIT algorithm and program have been entirely reformulated to provide a more efficient procedure with enhanced theoretical properties, to permit objective hypothesis testing and provide confidence limits. This program is now being applied to immunoassays, estimation of molecular weights for proteins and nucleic acids, and to dose-response curves. The DETECT algorithm and program for analysis of pulsatile hormone release has been extensively tested and validated, and compared with other methods by means of receiver-operating characteristic (ROC) curves to evaluate the relationship between sensitivity and specificity. The DETECT algorithm was shown to provide optimal sensitivity and specificity for a wide range of conditions, e.g., peak height, shape, duration, frequency and signal/noise ratio. The algorithm for calculation of the instantaneous secretory rate has been improved with respect to efficiency. New methods have been developed to provide objective, statistically valid computer analysis of two-hormonal time series, to evaluate the degree of coincidence of pulses and estimate the lag period, if any, between the two series. This approach has been applied to clinical studies of LH, prolactin, beta-endorphin, and cortisol. New programs have also been developed in this Section to assist the analysis and interpretation of self-monitoring of blood glucose. These programs utilize data entered automatically from glucose meters equipped with memory and clock-calendar to avoid the tedium of manual data entry. They enable one to visualize the circadian glucose profile in dynamic display ("glucose profile movie"), provide a report of hypoglycemic episodes, and allow visualization of the profile of insulin action in relation to the average glucose profile. These programs are now undergoing clinical evaluation.

Rodbard, Munson, and their co-workers have developed a new program for optimization of design (e.g., number and choice of ligand concentrations) for ligand binding experiments. This program can handle simple cases (e.g., 1 ligand, 1 class of binding sites), and complex ones, e.g., multiple ligands, multiple classes of sites, dose response surfaces, and use of selective competitive ligands to block undesired classes of sites, i.e., "blocking experiments". This program has been tested, validated, and is now being applied to facilitate studies of dexamethasone binding in the hypothalamus (in collaboration with the Developmental Endocrinology Branch), and to studies of phencyclidine binding to receptors in rat brain (see below). In experimental studies of drug-receptor interaction, this group has investigated the binding of phencyclidine, N-allyl-normetazocine, and several related compounds, to receptors in membranes prepared from rat and guinea pig brain. Using specialized techniques developed in this laboratory for the design and analysis of such studies (e.g., the programs DESIGN and LIGAND), these workers are able to demonstrate two distinct classes of binding sites for phencyclidine in rat and guinea pig brain. Both of these sites are distinct from the "sigma" site for (+)-SKF 10,047. Further, in the guinea pig, the "sigma" site appears to exist in both high and low affinity forms. This group has developed assay conditions for each of the four types of sites (PCP_1, PCP_2, σ_1, σ_2) and evaluated the rank order of potency of a large number of drugs. The functional significance of these receptor subtypes will be the subject of future investigations. In collaborative studies, Rodbard et al. have continued to characterize the receptors for vasopressin and oxytocin in the male and female genital tract in several species, including man.

The Section on Metabolism and Mass Spectroscopy, led by **Alfred Yergey**, has pioneered the development of thermal ionization mass spectroscopy, to permit the clinical investigation of calcium metabolism in full term and premature neonates, infants, children, puberty, pregnancy, lactation, aging, osteoporosis, and a variety of disease states. Further, this group has developed liquid chromatography - mass spectroscopy methods for the study of the kinetics of metabolism of glucose, cortisol, testosterone, progesterone, Vitamin D and other compounds in man.

This section has made important advances in the application of mass spectrometry to the identification of molecules and elements of biological and clinical interest, as well as in studies of the metabolic kinetics of several of these materials. (Stable isotopes of carbon, hydrogen, calcium and magnesium can best be measured at low levels by mass spectrometry, and are now routinely used as tracers or measurement standards). A major focus of the activity of this section is the elucidation of the in vivo metabolism of calcium. In several clinical investigations, two distinct calcium isotopic tracers are typically administered simultaneously, one orally and one intravenously. The time-dependent dilution of these by natural calcium is determined by thermal ionization mass spectrometry. These measurements are analyzed using multicompartmental mathematical models. Determination of the fraction of dietary calcium absorption can now be obtained using a two-day protocol instead of a lengthy and costly metabolic balance study. Application of this method to studies of normal and osteoporotic women, in collaboration with workers at the Mayo Clinic, has shown that there is no age dependence for absolute calcium absorption, although the kinetics of absorption are age dependent. Studies presently underway with a group at the Medical University of South Carolina are examining the question of ethnic differences in fractional absorption. The results of calcium kinetic studies extending over six weeks have shown a number of intriguing observations directly relevant to the understanding of skeletal growth and development. First, it appears that the pool of calcium with the most rapid turnover includes a portion of bone, and that this pool reflects skeletal growth. In ten subjects, ages 2 weeks - 14 years, the pool size was strongly correlated with incremental growth rate taken from standard tables. Preliminary results from studies of patients with osteoporosis suggests that this pool size is reduced compared with normal subjects matched for age and sex. Second, the mean residence time for calcium has been shown to correlate closely with skeletal mass. As a consequence, this work on calcium kinetics may lead to a new method for estimation of the skeletal mass, without dependence on radioisotopes.

A second major focus of Yergey's group is the development and use of instruments and methods for the application of thermospray liquid chromatography/mass spectrometry (ThLC/MS). Two major findings have emerged from this work in the past year. First, daily cortisol production rate in normal subjects has been found to be 9.6 ± 2.3 mg/day (n=11) and 30.7 ± 9.3 mg/day in six patients with Cushing's disease. This work, performed in collaboration with the DEB, NICHD, suggests that the production rate in the normal subjects is approximately one-half the value presently accepted in clinical practice. This finding is consistent with the clinical observation that cortisol replacement therapy administered to adrenalectomized patients using the previous estimates of production rate tend to result in signs or symptoms of hypercortisolism. This work is currently being extended to studies of adolescents and the potential role of cortisol metabolism in the initiation of puberty.

A second major accomplishment is the development of methods to characterize small peptides produced by tumor cell lines, e.g., bombesin-like growth factors. Leucine-enkephalin, a model compound, has been added to culture media at concentrations similar to those expected for other peptides of this size. The peptide has been recovered and identified by use of successive chromatographic separations, enzymatic digestion and finally ThLC/MS. New methods and apparatus have been developed to improve the efficiency of recovery of peptides in thermospray mass spectrometry.

The Section on Macromolecular Analysis, led by **Andreas Chrambach,** has continued its studies of the physical chemistry of peptides, proteins, nucleic acids and viruses, and the development of new methods for their fractionation using polyacrylamide and agarose gel electrophoresis, isoelectric focusing, and related techniques. Emphasis has been placed on the relationship between log-mobility and gel concentration (Ferguson plot), and its interpretation in terms of molecular or particle size, conformation and net charge. New theoretical analyses and computer programs provide quantitative interpretation of non-linear Ferguson plots, and can compensate for incomplete polymerization or abnormalities of gel crosslinking. The dependence of mobility on field strength and duration of electrophoresis have been investigated, and necessary correction factors have been measured. A pore-gradient gel, combined with time-lapse photography, has been used to simplify the construction of Ferguson plots.

An improved compartmentalized thin-layer slab gel apparatus has been designed for use with dried agarose and acrylamide gels in discontinuous buffer systems. In other recent work, an immobilized pH gradient has been used to achieve steady-state isoelectric focusing, and a series of technical problems has been resolved to make this a practical method, suitable for use in two-dimensional macromolecular mapping. Several of these new methods are being applied to the study of DNA-protein interactions. They are also being applied to the characterization of immunogens for vaccines for H. influenzae meningitis, in collaboration with the Laboratory of Developmental and Molecular Immunity. These studies are designed to examine the relationship between physical properties (aggregation state, net charge) and immunogenicity.

LABORATORY OF THEORETICAL AND PHYSICAL BIOLOGY

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Human Genetics Branch

William A. Gahl, M.D., Ph.D., Acting Chief

Top row: I. Owens, A. Mukherjee, E.J. Butler
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This Branch conducts research to elucidate the pathophysiology of human genetic and developmental disorders through an understanding of basic biological mechanisms. Clinical research projects include studies on the natural history, treatment, and methods of diagnosis of several heritable disorders of man. Basic research involves the use of in vitro systems to study aspects of cell biology with immediate or potential applications to human genetic diseases.

The Section on Human Biochemical Genetics, under the direction of **William Gahl**, studies a wide range of inborn errors of metabolism from both clinical and basic research aspects, and emphasis is placed upon investigations into lysosomal storage. There are two known storage diseases due to defective transport of small molecules out of lysosomes. The first is cystinosis, a multi-systemic disease of children resulting in kidney failure by age 10 years. Members of the Section care for 35 pre-renal transplant patients, offering the cystine-depleting agents cysteamine and phosphocysteamine as efficacious preventive therapy against impaired growth and renal function in these children. Cysteamine eyedrops are also being successfully studied to reduce the number of corneal crystals in affected patients. Cysteamine and phosphocysteamine are offered to approximately 20 post-renal transplant patients in whom non-renal complications of longstanding cystinosis are being described. These include exocrine and endocrine pancreatic insufficiency and ophthalmic and renal involvement. One 22 year old patient suffered from a myopathy with muscle cystine crystals and represented the first documented example of clinical muscle impairment in cystinosis. Members of the Section also described the first pregnancy in a cystinosis patient with a normal delivery and a normal infant despite cystine crystals in the maternal portion of the placenta. In a different infant affected by cystinosis, cysteamine therapy from two weeks of age attenuated the severity of the renal Fanconi syndrome, a tubular reabsorption defect, which is part of the clinical presentation of cystinosis. The same syndrome results in urinary loss of the nutrient carnitine, which is prescribed to our patients as replacement therapy. Blood carnitine levels are rapidly restored to normal by this treatment.

The second known lysosomal transport disorder is Salla disease, a Finnish disorder in which free sialic acid is stored in cellular lysosomes. In 1986, members of the Section demonstrated that the basic defect of Salla disease (and the more severe variant, infantile free sialic acid storage disease) is impaired transport of free sialic acid out of the lysosomes. This resulted in the receipt of several cell lines from patients with other disorders of sialic acid metabolism, and these defects are being aggressively investigated. In particular, two cell lines store free sialic acid in their cytosol, not their lysosomes.

In a basic research pursuit involving lysosomal transport of small molecules, members of the Section have described a carrier-mediated transport system for monoiodotyrosine (MIT) in rat thyroid cells in culture. MIT, a product of thyroglobulin hydrolysis in lysosomes, is a small molecule whose iodine needs to be salvaged by the cell for reincorporation into thyroglobulin and subsequent production of thyroid hormone. This iodine reutilization takes place in the cytosol, and the newly discovered lysosomal transport system for MIT explains how MIT's iodine travels from lysosome to cytosol for salvage. An impairment of this system in humans may result in iodine or thyroxine responsive hypothyroidism. Gahl and his colleagues have also recently become involved in investigating the cause of "new" storage diseases. They have collected several fibroblast lines from patients with an unidentified lysosomal storage disorder. Lysosomes are being isolated from fibroblasts and their contents analyzed for amino acids by ion exchange chromatography, sugars by pulsed amperometric detection, and lipids by thin layer chromatography. Later, liquid chromatography-mass spectrometry and electron probe analysis will be applied to the analysis.

A clinical protocol for the study of Lowe (oculocerebrorenal) syndrome has been established. The renal, ophthalmic, neurological, and joint manifestations of the disease are being studied, and optimal treatment modalities are planned. One of the Section's patients has been found to have central demyelination and peripheral neuropathy and other patients are being examined for these complications. Involvement of heterozygote mothers in the X-linked disease is also under investigation. The continued investigation of sulfur metabolism in a man with methionine adenosyltransferase deficiency has also been extremely illuminating. Sulfur and methyl balance studies in this individual demonstrated that in vivo, S-adenosylmethionine regulates the partitioning of homocysteine between degradation to inorganic sulfate and remethylation to methionine. In addition, transamination was shown to provide an active though minor pathway for methionine metabolism in the human. Finally, a 2 year old boy was diagnosed by workers in the Section as having a hepatic copper storage disease which, by clinical, histological, and biochemical criteria, resembles Indian Childhood Cirrhosis. The boy's fibroblasts are now being studied in an attempt to determine the cell biological causes of copper storage in this disease and to discover how cells handle copper in general.

In a separate project, **Jean Butler** studies the lysosomal storage of cholesterol in mutant cells, in collaboration with other NIH scientists. The exact causes of two lysosomal storage disorders, Niemann-Pick Disease Types C and D, remain unknown. It has now been demonstrated that the fibroblasts from the Niemann-Pick Type C patients store cholesterol in their lysosomes and fail to esterify the cholesterol. Niemann-Pick Type D fibroblasts also fail to esterify cholesterol, but do not manifest lysosomal storage of cholesterol. These discoveries are expected to help elucidate the biochemical defects in these disorders, and to reveal new aspects of cellular cholesterol metabolism.

Adjunct Scientist James Sidbury has continued to care for patients with glycogen storage disease and to study the disorder at the bench. He has demonstrated variability in the ability of affected children to hydrolyze a standard load of starch. Among several types of starch tested, corn starch remains the starch hydrolyzed in the most consistent fashion, and its long-term use in maintaining blood glucose concentrations in patients with glycogen storage diseases is now being studied. To date, the metabolic regulation that accompanies corn starch therapy has not resulted in a reduction in the size or frequency of the hepatomas which characterize type 1 glycogen storage disease in post pubertal patients. An important finding of the past year has been the discovery that 80% of post-pubescent patients with type 1 glycogen storage disease have glomerulosclerosis. This natural history information has important prognostic and therapeutic implications, among them the fact that renal disease should serve as a measure of the efficacy of dietary and pharmacological interventions. Another active effort involves determination of the rate of maximum glucose production in patients with hepatic glycogenosis. Stable isotope studies have revealed a rate of 2 gm/Kg body weight, although these results may not apply for very young children.

Adjunct Scientist Joan Caddell studies the audiogenic seizure-shock episode in weanling rats with acute magnesium deficiency as a model system for Sudden Infant Death Syndrome (SIDS). The rats show capillary aggregates of platelets, leukocytes, erythrocytes and lymphocytes in the lung and heart. The seizure-shock episodes could be almost completely aborted by administration of a thromboxane A2 receptor antagonist. (Magnesium deficiency is associated with the release of thromboxane A2 which causes platelet aggregation.) Work on this model system may broaden our knowledge of the physiological consequences of magnesium deficiency in animals and man.

A group headed by Joan Marini, of the Section on Molecular Biology, studies osteogenesis imperfecta (0I) and related bone diseases from molecular, biochemical and clinical perspectives. On the molecular level, the group has detected 5 point mutations in Type I collagen mRNA by developing a system for identifying mismatches in RNA/RNA hybrids using RNase A digestion. The point mutations are in patients with Types II, III, and IV 0I. Two Type IV mutations have been well localized using three overlapping probes near the 3' end of the alpha 1(I) cDNA. Sequencing of both alpha 1(I) alleles of one patient is being pursued using a cDNA library. On the biochemical level, overmodification of Type I collagen chains in Types II and IV 0I has been characterized using cyanogen bromide fragmentation of the chains in fibroblasts in chorionic villi. This approach, as well as investigation into proteoglycan and osteonectin production, is also being pursued in osteoblasts cultured in collaboration with Dr. Pamela Robey of the National Institute of Dental Research. Clinically, chorionic villus sampling is employed for the prenatal diagnosis of Types II and IV 0I by examining the collagen for defects. In studies of the endocrine parameters responsible for growth failure in 0I, nine of eighteen patients studied displayed a neurosecretory deficiency of growth hormone. Five children with 0I and poor growth are being treated with either clonidine or growth hormone itself; four have manifested improved growth. A lower limb bracing program for 0I patients continues to provide benefits to selected young children in terms of support and ambulation.

Marini's group also studies the treatment of fibrodysplasia ossificans progressiva, a disorder involving atopic bone formation, with the retinoid Accutane. They report a toxic effect of the drug on bone growth associated with the ability of retinoids to dedifferentiate chondrocytes. Surgical treatment of a jaw calcification has also been performed on a patient receiving Accutane and didronal. His clinical progress is being followed, and his tissue is now in culture for examination of its growth factor content, in particular EGF and TGF-ß.

Samuel Adeniyi-Jones, a member of the Section on Molecular Biology, continues to study the role of short repeated sequences (Alu-sequences) in the regulation of gene expression. His group has reported the identification of a 63 Kd protein in Xenopus oocytes which binds primary and processed transcripts of injected repeated sequence genes in both the nucleus and cytoplasm. Antibody to the protein inhibits Alu gene expression, and the protein appears conserved in evolution. Its specific role in Alu gene expression is under continued investigation.

The Section on Developmental Genetics, under **Anil Mukherjee**, conducts both basic and clinical research on the mechanism(s) of action and genetic regulation of endogenous steroid-induced antiinflammatory proteins. A fundamental question in biology is how an organism protects its epithelial lining from the external environment. Organs such as the tracheobronchial, gastrointestinal and genitourinary tracts come into contact with myriads of foreign antigens and yet, as a rule, do not respond with an inflammatory/immunological response. The mucosal epithelia of these organs secrete antimicrobial and antiinflammatory substances to modulate uncontrolled inflammatory/immunological reactions. Recently, Zasloff and others have described an antimicrobial defense system in vertebrates like Xenopus. However, the presence of an antimicrobial activity does not fully explain why there is an absence of inflammation, since dead microbes can still be antigenic to a host. For the past ten years these workers have suggested that small molecular weight proteins such as uteroglobin (UG) in the rabbit may be responsible for the modulation of an inflammatory response in the mucosal epithelium in mammals. Several workers have demonstrated, in mammalian species other than the rabbit, that proteins similar to uteroglobin also serve as modulators of the immune system. Therefore, this group compared the structures and amino acid sequences of three proteins, human lipocortin, rat seminal vesicular protein (RSV IV) and rabbit uteroglobin. A consensus sequence, a nonapeptide, was discovered; the synthetic nonapeptide proved a potent inhibitor of phospholipase A2 (PLA2) enzyme activity in vitro and an extremely potent antiinflammatory agent in vivo. In fact, the nonapeptide was more potent than indomethacin, ibuprofen and even dexamethasone in certain instances. These workers also discovered that the N-terminal fragments of Xenopus derived antimicrobial peptides form an amphipathic structure similar to the peptides derived from uteroglobin and/or lipocortin-l, and are potent PLA2 inhibitors in vitro and antiinflammatory agents in vivo. Thus, in Xenopus, the antimicrobial peptides degrade to produce antiinflammatory peptides. This explains the observation of Zasloff that the Xenopus wet epithelium neither gets infected nor inflamed even if the organism is allowed to live in a contaminated environment after surgery.

To delineate the physiological role of endogenous antiinflammatory agents ("antiflammins") large quantities of this protein are required. An inexpensive way to obtain this protein would be to use recombinant DNA technology to express uteroglobin (UG) in a bacterial host. Furthermore, expression of the UG gene in E. coli will allow site-directed mutagenesis studies to ascertain the function of this protein. Last year this group reported the construction of a plasmid cloning vector system for this purpose. Transformation of E. coli with those plasmids and induction with IPTG yielded UG but to a much lower level than required for preparative purposes. Therefore, this year reconstruction of this cloning vector was undertaken whereby the "trc" promoter controlling the transcription of the UG structural gene in the previous vector (pLE 101) is now replaced with an 89-bp DNA fragment controlling the "10" promoter of phage T7 and the translational start signal of the major T7 capsid protein. The resulting plasmid vector (pLE 103-1) was used to transform bacterial host BL21(DE3). These transformed bacterial cultures when induced with IPTG expressed 20-30 μg of UG per milliliter of bacterial culture. This high level of expression is adequate for preparative purposes. The recombinant UG thus produced appears to be a dimmer as secreted naturally by the endometrial cells of the rabbit when stimulated with progesterone. This appears to be the first demonstration that a protein with a quarternary structure such a UG can be produced in a bacterial host in its natural form. The successful development of this expression system paves the way for future site-directed mutagenesis studies on this protein. Moreover, this novel plasmid cloning vector has the potential to express complex proteins such as antibodies and other medically important substances.

Using cocrystallographic techniques in collaboration with Drs. Keith Ward and Virginia Pett at the Naval Research Laboratories, attempts are being made to study the possible interaction of the antiflammin peptides with the active site of PLA2. Preliminary studies using fluorescence of PLA2 active-site-tryptophan indicate that the peptides indeed interact with the active site tryptophan of PLA2. Confirmation of this and other observations will delineate the mechanism of action of these antiinflammatory peptides. Other studies on transformed cell lines from the endometrium and tracheobronchial epithelium are continuing. Two cell lines (RBE-7 and H5DC) have been fully characterized and found to secrete UG in vitro upon stimulation with progesterone. These cell lines provide a model system for investigating regulation of steroid action in vitro without the use of animal models; they provide a means to assess the biological potency of synthetic progestins and progestogens in vitro. (There is currently no in vitro system available to determine the biological properties of a steroid hormone.) Finally, it has been proposed that patients with cystic fibrosis have impaired arachidonate metabolism. The resultant increased arachidonate levels would increase tissue levels of eicosanoids (some of which are proinflammatory), perhaps leading to the profound inflammation observed in CF tracheobronchial epithelium and elsewhere. A preliminary collaborative study suggests a lack of UG-like immunoreactivity in cystic fibrosis epithelial cells compared with normal controls; this finding might explain the increased inflammation in CF. Using a UG cDNA probe, a human lung and prostatic expression library is now being screened for UG-like genes in humans.

The Section on Developmental Genetics is also studying the genetic factors predisposing to the development of fetal toxicity due to ethanol. During the past year, members of the Section have studied the rate of survival in thiamine deficient medium of cultured amniotic fluid cells derived from 10 pregnancies resulting in the birth of FAS babies and compared them to 12 normal control cell lines. These ongoing studies will delineate whether the high transketolase Km for thiamine pyrophosphate, observed previously in some of these cell lines, make them more sensitive to thiamine deficiency.

The Section on Cellular Differentiation, led by **Janice Chou**, conducts research to understand the regulation of gene expression during normal and abnormal differentiation processes. Several problems in gene regulation are emphasized, including expression of the alpha-fetoprotein (AFP) gene in liver and cloning and expression of the human pregnancy specific beta-1-glycoprotein (PSbetaG) gene.

Over the past several years, this group has studied expression of the AFP gene in fetal and adult rat livers. They found that the adult rat liver contains three AFP mRNAs of 2.2 (minor), 1.7 and 1.5 kb. These transcripts share a common 3' sequence, but the 1.7- and 1.5-kb AFP mRNAs lack sequences present in the first seven 5' exons of the 2.2-kb AFP mRNA. Sl nuclease analysis maps the 1.7-kb mRNA at the 5' boundary of the eighth exon of the 2.2-kb AFP mRNA and the 1.5-kb mRNA in the middle of the eighth exon. A cDNA clone (ARFP5) encoding the 1.7-kb RNA has been isolated from an adult rat liver cDNA library. The 90-bp 5' sequence of ARFP5 is not present in the 2.2-kb fetal AFP mRNA, although ARFP5 does contain nucleotide sequence present in the 2.2-kb AFP mRNA extending from the beginning of its eighth exon (nucleotide 873) to the 3' end. The 1.7-kb AFP mRNA found in adult liver is indistinguishable from a variant AFP mRNA identified by Chou and coworkers in a fetal liver cell line. However, the 1.7-kb RNA could not be reliably identified in fetal rat liver. The developmental profile of these AFP transcripts shows that fetal rat liver contains mainly the 2.2-kb mRNA which decreases to a very low level around the fifth week after birth. The 1.7- and 1.5-kb AFP mRNAs can be visualized about the 3rd week after birth, and the levels of these mRNAs increase to about 0.01% of the AFP mRNA level in 18-day-old fetal liver by the 5th week after birth. These two RNAs are the major AFP mRNAs in adult rat liver. Both the 1.7- and 1.5-kb AFP mRNAs are translationally active; they direct the cell-free synthesis of two polypeptides of 50 and 44K. This group has examined factors required to promote liver differentiation in vitro using primary fetal rat hepatocytes as a model system. They found that in the absence of effectors, primary fetal hepatocytes dedifferentiated. Cells maintained in the presence of glucocorticoid hormone or cAMP produced high levels of albumin and transferrin, or albumin and AFP, respectively. Both glucocorticoid and cAMP induced expression of adult liver-specific genes, suggesting that these fetal hepatocytes have matured. This study demonstrated that both glucocorticoid hormone and cAMP are necessary for optimal differentiation of fetal hepatocytes in vitro.

As an initial step towards a better understanding of the functions of human pregnancy-specific beta-1-glycoprotein (PSbetaG), the Section has isolated and characterized four cDNA clones (PSG16, PSG93, PSG95, and PSG9) encoding human PSbetaG. PSG16 (1.9-kb), PSG93 (2.1-kb), and PSG95 (2.2-kb) encode three polypeptides of 417, 419, and 426 amino acids with apparent molecular masses of 46.9, 47.2, and 47.8K, respectively; these three PSbetaG species diverge only at the 3' end of the coding regions (after amino acid 414). PSG9 (1.6-kb) encodes a polypeptide of 326 amino acids with an apparent molecular mass of 36.4K. In placenta, four nonglycosylated polypeptides of 50, 48, 46, and 36K have been identified by in vitro translation of placental poly(A)+ RNA. The apparent molecular masses of the native placental PSbetaGs are glycoproteins of 72 (major), 64, and 54K. Three PSbetaG mRNAs of 2.3, 2.2 and 1.7 kb can be detected by the four cDNAs identified. Thus, PSbetaG is heterogeneous at both mRNA and protein levels.

This group has found that the amino acid sequences of PSbetaG as deduced from the cDNA sequences of PSG16, PSG93, and PSG95 contain two repeated protein domains (la and 2a) of 93 amino acids each and a Ib domain. PSbetaG species encoded by PSG9 contains the la and Ib domains. PSbetaG shows strong homology to human carcinoembryonic antigen (CEA) at both nucleotide and amino acid levels. CEA contains eight domains including an N-terminal domain, three repeated domains (I, II, and III) each containing two subdomains (A and B), and a hydrophobic carboxylterminal domain. PSbetaG contains a CEA-like N-terminal domain, one or two repeated domains (a) similar to the A subdomains of CEA which is followed by a domain (Ib) similar to the B subdomains of CEA, but lacks a hydrophobic carboxyl-terminal domain. The positions of the cysteine residues in each domain are also conserved, indicating that PSbetaG and CEA are two members of the same gene family. The similarity between CEA and PSbetaG suggests that both proteins may play similar roles in growth control in development and differentiation.

An immunoreactive PSbetaG-like molecule has also been detected in human nonpregnant serum and fibroblast cultures, thus raising the question of the functional role of PSbetaG in pregnancy. This Section has demonstrated that although placental fibroblasts produced all three PSbetaG mRNAs of 2.3, 2.2, and 1.7 kb, the major PSbetaG molecule synthesized is a 62K variant PSbetaG, while the major placental species is 72K. Since cellular functions persisting *in vitro* are functions essential for growth, the difference in the major PSbetaG species between placenta and fibroblasts may be due to different functions of the various PSbetaGs.

Drug oxygenations and subsequent glucuronide conjugations are catalyzed by a membrane-bound multistep enzyme complex. These enzymes are important in the process of detoxification and elimination of many normal body constituents and many foreign chemicals taken in from our environment in the form of food additives, air pollutants, and medication. There are, as well, diseases of humans ascribed to deficits in drug-conjugating enzymes. Examples in pediatrics include neonatal hyperbilirubinemia, Gilbert's syndrome and the Crigler-Najjar syndrome. Teratogenesis and neonatal drug toxicities are thought to occur as a consequence of deficient glucuronidation of certain drugs, such as dilantin and chloramphenicol. Therefore, experimental model systems were developed to improve our understanding of the molecular regulation and neonatal development of the various UDP-glucuronosyltransferases (UDPGT). These model systems are being used particularly in the study of the basic enzymology and cell biology of glucuronidation, and to develop a definitive diagnosis by recombinant DNA techniques of the Crigler-Najjar Syndrome in affected neonates. The Section on Drug Biotransformation, under **Ida Owens**, which in previous years cloned and sequenced the mouse $UDPGT_{m-1}$ gene, has carried this characterization a step further by determining the hydropathy profile of the encoded enzyme. N- and C-terminal sequences were found which are consistent with membrane insertion and membrane spanning sequences. It is difficult to study the biochemistry of any one form of this enzyme by conventional biochemical techniques because the various forms are not easily separated from one another. Transfection of the cloned mouse gene is therefore the preferable approach. The group has succeeded in transfecting this gene into yeast strains, where it encodes a membrane bound enzyme of about M_r 51,000, and has determined its substrate specificities. The enzyme becomes cytosolic and its substrate specificity changes markedly when the cloned gene is inserted without its putative membrane insertion signal sequence at the N-terminus. This year a human UDPGT gene was completely sequenced. It has been successfully expressed in yeast microsomes and the substrate specificity of the encoded enzyme is being determined. The human gene showed polymorphism when compared with that of another published sequence; other polymorphisms are being sought at present using lymphocytes and liver biopsies in order to determine whether there is a correlation between polymorphism and substrate specificity. Studies on mRNA isolated from a Crigler-Najjar patient showed reduced hybridization to the human gene compared with its own transcript. Work is in progress to determine the precise genetic defect in this heritable disease.

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Cell Biology and Metabolism Branch
Richard D. Klausner, M.D., Chief

Left to right: J. Harford, R. Klausner, W. Leonard, L. Samelson

This Branch is broadly concerned with the areas of iron metabolism, gene regulation, receptor biology, and immunology. The work of the laboratory has been divided between four projects involving the cellular and molecular biology of human iron metabolism, post-transcriptional mechanisms of human gene regulation, and receptors involved in initiating and regulating the immune response. During the past year, the Branch also initiated a study on the cell biology of the membrane proteins encoded by the human immunodeficiency virus (HIV), the etiologic agent responsible for AIDS.

The long term interest of this laboratory in understanding the basis of iron metabolism is reflected in the groups led by **Joe B. Harford** (Biology of the Transferrin Receptor) and **Richard D. Klausner** (Ferritin and the Clinical Basis of Iron Metabolism). The importance of iron metabolism in human biology becomes apparent in many areas of research. Because iron is absolutely essential and perhaps rate limiting in the growth of cells, the ability of cells to regulate and assure iron uptake is strictly correlated with their proliferative rate. This is true during development, in normal differentiation, proliferation, inflammation, the immune response, wound healing and neoplasia. In addition to its correlation with cellular growth, iron is absolutely required for maintenance of baseline metabolic activities. Thus, obtaining sufficient iron is critical for the health of both the individual cell and the entire organism. This importance is reflected in the wide range of clinical consequences of iron deficiency, a common human disorder. In contrast to iron deficiency, where insufficient iron leads to pathologic consequences, too much iron is extremely toxic and will lead to cell death. The inability to regulate the uptake of iron underlies one of the most common genetic diseases of man, hereditary hemochromatosis. This disease, which affects one in 400 people in our population, is due to a failure to regulate iron uptake and results in a tremendous amount of morbidity and, if untreated, mortality. The Cell Biology and Metabolism Branch is at the forefront of studies aimed at elucidating the molecular basis of iron metabolism. Studies by this group continue to focus on two genes, and the current interest is on how these genes are regulated by iron. These workers are emphasizing this aspect of iron metabolism because it is the ability of iron to be regulated in a homeostatic manner that underlies the cellular mechanisms that are designed to prevent both iron deficiency and iron toxicity. We now know that there are two critical elements that underlie the iron regulatory system: The transferrin receptor, along with its ligand, transferrin, and ferritin. Work from this Laboratory has brought us very close to a molecular understanding of how these genes are regulated in response to iron. Ferritin, the protein which sequesters iron, making it unavailable for cell metabolism, is regulated at the translational level such that when there is little iron there is very little translation, and when there is a large amount of iron there is a larger amount of translation. The transferrin receptor, which permits the entry of iron into the cell, is predominantly regulated at the level of the half-life, of the mRNA encoding the protein. In contrast to ferritin, when there is little iron, mRNA levels for the transferrin receptor increase due to a longer half-life, and when there is more iron the half life of the mRNA shortens, resulting in a drop in receptor number and less iron uptake into the cell.

Work in this Laboratory over the past several years has allowed these investigators to define two molecular elements underlying the regulation of these two genes. One is an RNA element, a sequence found within the messenger RNA molecules encoding both ferritin and the transferrin receptor. This element, which Klausner et al. refer to as an iron-responsive element or IRE, is a small stem-loop structure which endows the regulation of the fate of the RNA with iron sensitivity. A single IRE present in the 5' untranslated region (5' UTR) of ferritin mRNA molecules is entirely responsible for the ability of iron to regulate the translation of the ferritin message. In fact, this element has been isolated and can be inserted into the 5' untranslated region of any gene and thereby confer iron-dependent regulation of translation upon that gene. The regions of the mRNA of the transferrin receptor responsible for its regulation in response to iron are contained within the 3' untranslated region. This region has been localized and analyzed and has been shown to contain five RNA motifs with sequences resembling the ferritin IRE. That the sequence motifs in this region of the transferrin receptor are in fact capable of being functional IREs has been demonstrated by cloning these sequences into the 5' UTR of reporter genes and demonstrating that they can confer iron-dependent translational control (identical to that seen in ferritin) to these exogenous genes.

The second molecular element involved in the regulation and expression of these iron related genes is a cytosolic protein (proteins) which specifically binds with high affinity to the IRE RNA regulatory element. This protein has been identified by the use of a combination band shift and RNAse protection assay. As predicted from sequence analysis, the same cytosolic binding protein interacts with the IREs found in the ferritin 5' UTR and with IREs identified in the regulatory region of the 3' UTR of the transferrin receptor. These observations have allowed this group to formulate a unifying model to explain the apparent disparate regulation of these two genes. According to this model, when there is little ambient iron, the IRE binding protein becomes activated and binds to the IRE. If the IRE is in the 5' UTR of the ferritin gene, the binding of that protein will sterically block the initiation of translation. For the transferrin receptor mRNA, the situation is a bit more complex. In order to explain the regulation of message half-life by iron via the IRE and its interaction with the binding protein, the following can be proposed: Once again, the absence of iron activates the IRE binding protein to bind to the RNA regulatory element. These workers propose that near the IRE, in the 3' UTR of the transferrin receptor gene, is a site which is a recognition sequence for attack by an endoribonuclease. Attack by this endonuclease results in a short half-life of the receptor mRNA. However, when the neighboring IRE is occupied by the IRE binding protein, access to this site is blocked, the message is not degraded, and message levels rise. In this way, the increased binding of the IRE binding protein in the absence of iron can explain both the decreased rate of biosynthesis of ferritin and the increased rate of biosynthesis of the transferrin receptor. Support for this model has been provided by the finding that when cells are treated with an iron chelator in order to starve them of accessible iron, the specific activity of the IRE binding protein in the cytosol is considerably increased. In addition to this model for how the IRE binding protein could regulate iron-responsive genes at the post-transcription level, this group is beginning to unravel the actual biochemical mechanisms by which the IRE binding protein may function vis-à-vis iron levels and in its interaction with the IRE.

In addition to the transferrin receptor, transferrin and ferritin, other critical genes are undoubtedly involved in human cellular and total body iron metabolism. The identification of these genes, however, is a major problem. Because the essentials of iron metabolism are likely to be similar in all organisms, be they primitive prokaryotes, yeast, or higher eukaryotic and mammalian cells and organisms, the Branch has embarked on a new approach to solving this problem. Accordingly, these investigators are examining iron metabolism in the yeast Saccharomyces cerevisea. They have shown that this organism is absolutely dependent on the uptake of environmental iron for health, growth and proliferation, and are using biochemical, protein chemical and, most importantly, genetic approaches in order to identify the genes and gene products of this yeast that are involved in iron uptake. In particular these workers are focusing on an iron reductase gene present in the membrane of yeast cells that is probably responsible for the initial events in the transport of iron across cellular membranes. This is one of the major gaps in our understanding of human iron metabolism, and obtaining the yeast gene encoding this iron reductase should allow identification of the corresponding higher eukaryotic gene.

One of the goals of all of these studies is the application of the insights gained to diseases of iron metabolism. The Branch continues to have a very active clinic in which patients with hereditary hemochromatosis are studied and continuous cell lines are established from their peripheral blood lymphocytes. This provides the Branch not only with cells from these patients with which to study physiologic abberations, but also with a source of genetic material to study the molecular basis of possible defects underlying hereditary hemochromatosis. In addition, these studies are providing unique insights into specific mechanisms of gene regulation in human cells, and they are also offering useful applications of recombinant DNA technology. Accordingly, the discovery of the IRE has led to the patenting of this new type of genetic element for possible use as part of an iron-regulated expression vector system.

Largely due to the work of this Laboratory, the T cell antigen receptor is now understood as one of the most complex integral membrane receptor molecules. This complexity applies both to subunit structure and biochemical function. We now know that the receptor is composed of at least seven different proteins that are assembled in complexes consisting of seven, nine or more chains. This work is directed by **Richard D. Klausner** (Structure, Function and Cell Biology of the T Cell Antigen Receptor) and **Lawrence E. Samelson** (The T Cell Antigen Receptor and Immune Activation) who have named the two most recently defined chains of this receptor complex, the zeta and eta chains. Both the murine and the human zeta chain genes have been cloned by these investigators in the past year and this has led to a complete elucidation of the structure of the proteins. The zeta chain occurs in two different forms, as a homodimer and as a heterodimer. In the latter situation it is linked to a somewhat larger chain which has been called eta. The frequency of eta is only about one-tenth the frequency of zeta and thus the heterodimer is most likely only found in the minority of surface receptors. One of the goals of these studies is to correlate the structure of the receptor with its function, defined by examining both the very proximal biochemical events that ensue upon receptor stimulation or the resulting phenotypic changes in cells (generally referred to as cell activation). In order to make these structure/function correlations, two approaches are taken: 1) The isolation of mutants or variants of antigen-specific T cell hybridomas; and 2) the reintroduction of genetically altered T cell receptor subunits into deficient T cell lines. Over the past year much progress has been made with the first approach. In particular, functional consequences of failing to synthesize either the zeta or the eta chain have been examined. The absence of the eta chain has no effect on the surface expression of the receptor complex. However, it does seem to be correlated with severe functional deficiency. This group has previously shown that the T cell antigen receptor functionally couples to at least two cellular biochemical pathways. In one, phosphorylated phosphatidylinositides are broken down in response to receptor activation, leading to the release of water soluble inositol phosphates and diacylglycerol. The result of this is the activation of protein kinase C and the mobilization of intracellular calcium. The other pathway involves the activation of a non-receptor tyrosine kinase (or tyrosine kinases) which results in the tyrosine phosphorylation of a number of cellular substrates (see below). In the absence of eta, receptor-mediated tyrosine kinase activation is maintained while the ability to couple the phosphatidylinositide pathway is lost. The eta chain is made in such small quantities that it is likely that only a subpopulation of T cell receptors contains this chain. Despite this fact, this population is critically correlated with one pathway that leads to full T cell activation. The functional importance of this minor chain of the T cell receptor makes it imperative that we clearly define exactly what this chain is. Recent data has finally allowed these workers to determine that it is structurally related to the zeta chain, and they are now attempting to determine genetically the nature of this relationship.

As stated above, multiple kinases are activated in response to the stimulation of the T cell antigen receptor. An understanding of the nature of these kinases, their pattern of activation, their pattern of regulation, their interaction, and their relevant cellular substrates would be absolutely critical to our ability to understand and possibly manipulate the immune response. These workers have defined a set of cellular substrates that are tyrosine phosphorylated in response to receptor activation. Recent data suggests that there may be two different sets of substrates phosphorylated in response to receptor occupancy and that these two sets may be the targets of two different tyrosine kinases. One set includes the rapid phosphorylation (within seconds) of a cytosolic protein called pp62. After pp62 is phosphorylated, the zeta chain of the T cell receptor is phosphorylated. Another set of substrates has been defined which appear to be extremely susceptible to dephosphorylation by a tyrosine phosphatase within the cell. In order to observe the phosphorylation of this set of substrates, one needs to examine the cell in the presence of the tyrosine phosphatase inhibitor. The pharmacologic characteristics of the phosphorylation of these two sets of substrates are quite distinct. This group is currently attempting to purify the pp62 tyrosine kinase substrate as it may be the tyrosine kinase linked to the T cell receptor. In response to receptor stimulation an additional cytosolic kinase is phosphorylated. This is the cellular homologue of the raf-oncogene. This kinase is a serine/threonine specific kinase and every molecule of c-raf within the T cell is phosphorylated, perhaps on multiple sites, in response to receptor activation. This group is currently exploring the regulatory consequences of the phosphorylation of this proto-oncogene.

In addition to studying the structure and function of the T cell antigen receptor, these investigators have been studying the cell biology of this receptor. In particular, they are attempting to understand the mechanisms and pathways by which newly synthesized receptor chains are assembled with each other and finally expressed on the cell surface. This has been an extremely productive area in the past year, leading to important new concepts in cell biology which will have wide-ranging implications and applications. These studies have provided the most complete picture to date of the process whereby the cell assembles multicomponent complexes within the membrane system. Using the seven chain T cell antigen receptor complex, the Lab has examined the concept of architectural editing whereby the three-dimensional structure of newly synthesized and assembled membrane protein complexes are recognized by the cell as either being correct or incorrect. Following this recognition the fate of these complexes within the cell is determined, and multiple fates can befall these complexes. When the complete and correct complex is formed within the endoplasmic reticulum (ER), it is transferred through the Golgi apparatus, and finally expressed on the cell surface. Certain incomplete complexes are transferred out of the ER through the Golgi but are rapidly delivered to, and destroyed within, the lysosomes. Finally, the majority of incorrect complexes formed are retained within the endoplasmic reticulum system, never reaching the Golgi apparatus. Interestingly, within the endoplasmic reticulum these complexes are degraded. Characterization of this ER degradation has revealed an entirely new degradative system, non-lysosomal in nature. This degradative system is exquisitely sensitive to cytosolic pH, suggesting that it may be regulated by hormones, etc. One feature of this degradation is that different proteins show vastly different rates of susceptibility to this degradation. One principle underlying differential susceptibility is the state of assembly for particular proteins. Thus an assembled subunit of the receptor complex has a relatively long half-life within the endoplasmic reticulum while the free subunit is rapidly destroyed. Retention and rapid degradation within the endoplasmic reticulum system explains a wide variety of phenotypic changes that accompany spontaneous human mutations. For example, in a variety of forms of familial hypercholesterolemia, the LDL receptor is present in extremely low amounts within the cell. The explanation for this may be retention and degradation in the endoplasmic reticulum. A variety of other examples of ER degradation exist, and these workers are currently pursuing both the physiologic and pathophysiologic roles of this new pathway described in this Laboratory. The early stages in the assembly of a multicomponent complex within the endoplasmic reticulum are extremely complex, and they have not been described in detail for any system. These scientists are beginning to unravel both the kinetics and mechanism of this assembly. In particular, they are interested in a protein that they have described, called TRAP (T cell receptor associated protein) which non-covalently assembles with the newly synthesized chains in the endoplasmic reticulum. TRAP stays with these chains for about 15 minutes while they assemble, and then TRAP dissociates. The function of this new protein is unknown, but it may be to catalyze correct assembly of these many chains with each other. Purification, characterization and molecular cloning of the gene encoding TRAP will be of great importance in understanding the fundamental cellular process of multi-chain assembly. Such work is ongoing within the Laboratory.

Having described the complicated pathways for newly synthesized membrane proteins in T cells, Klausner and his colleagues turned their attention to an attempt to describe similar pathways for the envelope glycoproteins of the human immunodeficiency virus, HIV. Little work has been done on the cell biology of assembly of this important human pathogen. In collaboration with Malcolm Martin and Ron Willey, NIAID, this group has described the pathway taken by the newly synthesized envelope glycoprotein of HIV, which is encoded by a single gene. It is inserted into the endoplasmic reticulum, transferred relatively slowly out of the ER into the Golgi, and at some point late in the Golgi or immediately thereafter, it is cleaved by an acid dependent protease to the two glycoproteins that make up the membrane proteins of the infectious virus. Interestingly, only 5-10% of the precursor glycoproteins are ever cleaved. The uncleaved glycoprotein is efficiently transferred to lysosomes where it is degraded, while the cleaved glycoproteins are efficiently spared from lysosomal degradation and instead are released from the cell, presumably as viral particles. This work has provided the basis for attempts to design new drugs to inhibit the (inefficient) processing of the glycoprotein. This processing is absolutely essential for the production of infectious particles. These workers have shown that this process is exquisitely sensitive to membrane permeable amines, and are now examining whether such bases would be valuable in interfering with HIV infection. This work represents a clear example of the applicability of studies aimed at understanding the basic cell biology of membrane proteins to human disease.

T cell activation is accompanied by a genetic program whereby a number of genes are turned on in a predictable and defined pattern. This pattern of genetic program expression is stereotyped both in terms of kinetics, order of genes, and the groups of genes involved. One of the most important sets of genes that are turned on involves the interleukin-2 system, which is the focus of a group led by **Warren J. Leonard**. Interleukin-2, or T cell growth factor, provides autocrine, paracrine and endocrine stimulation for the proliferation of T cells. In order for these T cells to respond to this growth factor, they must express a receptor that is specific for it. The IL-2 receptor group has continued to examine two aspects of its biology. The first of these is directed towards determining the genetic elements that are involved in the carefully regulated expression of the interleukin-2 receptor alpha subunit gene. Using a variety of techniques for the study of 5' genomic flanking elements as transcriptional elements as well as techniques aimed at looking at specific DNA protein interactions, this group has come a long way in elucidating the sequences involved in the expression of this gene. In addition to the regulated expression of this gene during the activation of T cells, the gene for the IL-2 receptor alpha chain is also highly expressed in an apparently unregulated fashion in cells infected with and transformed by the human T cell leukemia virus, HTLV-I. Again, this group has been defining those elements that appear to be responsive to the expression of gene products encoded by this virus. One area of particular interest over the past year is the potential role of a particular DNA binding protein, first described as an enhancer element for the kappa immunoglobulin light chain gene. A consensus sequence that defines this element binds a particular transcription factor called NF-kappa-B. It appears that the same motif is present and capable of binding an NF-kappa-B molecule (or molecule closely related to it) in the regulatory region of the IL-2 receptor gene. The role of this transcriptional factor in the expression of the IL-2 receptor alpha chain gene is being explored. This is of particular interest because of the role of these sequences and this factor in the activation of the human immunodeficiency virus, HIV.

Another aspect of the biology of the interleukin-2 receptor is the role of the beta subunit of the receptor (p70), first described by this group two years ago. The ability of the beta subunit to exist on the cell surface in the absence of the alpha subunit and to serve as a receptor in those cells has been demonstrated. This is true of a variety of peripheral blood mononuclear cells such as large granular lymphocytes and the precursors of LAK cells and NK cells. That the beta subunit can be a receptor, or part of a receptor, in the absence of the alpha subunit is demonstrated by both its ability to bind IL-2 and, in fact, mediate biologic responses to that binding. The beta chain of the IL-2 receptor is not restricted to T cells but can be induced to exist on the surface of both peripheral blood B cells and monocytes. The potential roles in immunobiology of this receptor are only beginning to be explored. This group has made considerable progress in purifying the beta subunit of the IL-2 receptor which is an essential step towards the molecular and molecular genetic characterization of this critical protein of the immune system.

Finally, this group has been engaged in characterizing other genes that are rapidly turned on in response to T cell activation. One of these, which has been referred to as Act-II, is rapidly turned on in both T cells and other mononuclear cells in response to stimulation. The gene and cDNA encoding this protein have been characterized. The cDNA predicts a small secreted protein, suggesting that this may be a new lymphokine. In addition, this protein has recently been expressed in a variety of systems including a bacculovirus expression system in cultured insect cells. This system has allowed the secretion of a large amount of protein to enable detailed biochemical analysis as well as functional studies of this novel T cell activation gene product.

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Laboratory of Developmental and Molecular Immunity

John B. Robbins, M.D., Chief

Left to right: E. Hanna, J. Robbins, R. Schneerson, S. Szu, K. Ozato

The period from embryogenesis to childhood is a time of extraordinary development during which the expression of immunity changes quickly, eventuating in the adult expression of immunity with its complex regulatory mechanisms. The basis for this development and the relation of incompletely expressed immune elements to the acquisition of infectious diseases has been the object of study by this Laboratory.

The Section on Bacterial Disease Pathogenesis and Immunity, led by **John Robbins**, has been concerned with the specific bacterial products responsible for virulence and the host immune mechanisms involved in the prevention of common and serious bacterial infections of newborns and young children. Robbins and his colleagues **Rachel Schneerson** and **Shousun Szu** have focused on a series of invasive diseases due to encapsulated bacterial pathogens of which Haemophilus influenzae type b is the most common. The mechanism of virulence and the protective antigen of this pathogen are associated with its capsular polysaccharide. Synthetic schemes have been developed by these workers to convert the capsular polysaccharide of H. influenzae type b into an immunogen capable of inducing protective levels of antibodies in infants by covalently binding it to a protein. A conjugate vaccine, composed of the influenzae type b capsular polysaccharide chemically bound to tetanus toxoid, has been tested and shown to be capable of inducing protective levels of antibodies in infants after the second injection (concurrently with DTP). The third injection induces the same level of antibodies as in immune adults. Passively acquired antibodies to the H. influenzae type b capsular polysaccharide and to the tetanus toxoid induce antigen-specific suppression despite the fact that both components are on one molecule. Effectiveness studies to document the ability of this new vaccine to prevent H. influenzae type b meningitis and other related serious diseases of infants and children are underway. The same technology has been applied to pneumococcus type 6 and pneumococcus type 12 capsular polysaccharides, and studies to evaluate the effectiveness of these new vaccines, especially in infants and children with sickle cell anemia, are also underway.

Another serious invasive disease, caused by encapsulated bacteria, is typhoid fever. Two controlled double-blinded field trials undertaken by this group have verified the protective effect of the Vi capsular polysaccharide in preventing typhoid fever. The immunogenicity of the Vi has been improved by covalently binding it to a T cell-dependent carrier protein. In this case, a conjugate of Vi-tetanus toxin has been synthesized and its immunogenicity, safety and protective activity are being studied in Nepal. The notion that serum antibodies can prevent invasive diseases caused by enteric bacteria has most recently been directed toward non-typhoidal Salmonellae and to Shigellae. The 0-specific side-chains of these two bacteria have been purified, detoxified, and successfully bound to the beta-subunit of cholera toxin and the beta-subunits of the Shigellae toxins. Clinical trials to verify the safety and efficacy of these new vaccines are underway. Bacteremic disease due to Staphylococcus aureus remains a serious and common problem of patients in hospitals. It has been discovered that S. aureus has capsular polysaccharides that are covalently bound to the bacterial cell wall, and which have unusual chemical properties that require new serologic methods for their detection and new chemical processes for their isolation and characterization. Antibodies to these capsular polysaccharides have been shown to facilitate in vitro phagocytosis. Conjugates, composed of the S. aureus type 8 and type 5 capsular polysaccharides chemically bound to Pseudomonas aeruginosa exotoxin A, have been synthesized and these two conjugates are planned for clinical trials. It is hoped that passive immunization of vaccine-induced antibodies to these capsular polysaccharides may prevent bacteremic S. aureus disease in hospitalized patients.

A new vaccine, developed by this group and composed of pertussis toxin inactivated by a novel reagent for preparing vaccines, hydrogen peroxide, has been studied in adults and children. The purified toxoid has been characterized by physical, chemical as well as biologic means and was shown to be biologically inactive and pyrogen-free. Metabolic and clinical studies have shown that the toxoid is immunogenic and safe in adults and children. The levels of antibodies induced by the pertussis toxoid vaccine were comparable to or slightly higher than those observed in adults convalescent from the disease. Levels of antibodies in infants and children were higher than those induced by DTP vaccine. Clinical trials to assess the safety and immunogenicity of this pertussis toxoid vaccine in infants in Boston and in

Goteborg, Sweden are underway. It has been postulated that serum antibodies to this toxin confer antibacterial, antitoxin and antipertussis activity to the immunized host.

The Section on Immunoregulation and Cellular Control, directed by **Edgar Hannah**, has been studying the effects of bacterial toxins upon cloned lymphocyte hybridomas capable of expressing either precursor or mature T--cell regulatory phenotypes. Immune cytotoxic (CTL) cells have been studied similarly. Several bacterial toxins (including lipopolysaccharide endotoxin, streptococcal pyrogenic enterotoxin C, toxic shock syndrome toxin 1, pertussis toxin and streptococcal pyrogenic exotoxin) have been used to probe these precursor cells. The target for the action of these toxins has been studied by examining the effect upon antibody forming cells and cytotoxic T-cells incubated with these toxin treated precursors. Streptococcal pyrogenic exotoxin has been shown to result in decreased suppressor cell function. One of the mechanisms proposed is that the precursor regulatory T-cells, following treatment with this toxin, have lost their capacity to express interleukin-2 receptors. Streptococcal pyrogenic exotoxin was also observed to reduce permanently the amount of CD8, but not CD4, of double expressing (CD4/CD8) precursor clones. Similarly, pertussis toxin treated precursor cells result in a decrease in the cytotoxic T-lymphocyte response of mice. The cellular basis for these toxin-mediated effects upon precursor lymphocytes is under study.

The Section on Molecular Genetics of Immunity, directed by **Keiko Ozato**, studies major histocompatibility class I antigens, an essential component of protective immunity. These are a highly polymorphic group of cell surface components. Expression of these antigens varies according to developmental stage and type of tissue. Lymphokines, such as interferon and tumor necrosis factor, induce the expression of the antigens. The molecular basis for the regulation of MHC class I antigen expression has been studied at the molecular and cellular level by Ozato and her colleagues. The DNA sequences upstream of the MHC class I genes have been isolated and some of their functions that could exert regulatory activity have been studied. Two gene segments, in particular, have been identified. They are the conserved 5' upstream class I regulatory element (CRE) and the interferon consensus sequence (ICS) which are involved in both the developmental expression of and induction of class 1 MIHC antigens by interferon. Site-directed mutagenesis of these segments has been used to determine the precise sequence required for regulation. The activity of the mutant regulatory sequences has been studied by using the chloramphenicol acetyl transferase (CAT) assay.

The effect of protein binding to the CRE was studied by using undifferentiated F9 embryonal carcinoma cells which do not express MHC genes, and L-fibroblasts in which these genes are expressed. It was found that only two of three sequences in the CRE are bound by nuclear proteins extracted from F9 embryonal cells. In contrast, all three regions of the CRE were bound by nuclear proteins extracted from the L-fibroblasts. Protein binding to the CRE correlated well with the stage of development in that binding proteins are found only in tissues that express MHC class I genes. Mutants that have altered binding sites within the CRE failed to interact with specific binding proteins. Enhanced MHC class I gene expression was related to the binding to each of the three CRE regions. Binding of the nuclear factors to the interferon consensus sequence also exerts an effect upon expression of class I MHC genes. Interferon treatment induced binding of new proteins to this sequence, which correlated with enhanced MHC class I gene expression.

The c-fos oncogene, which encodes a DNA binding nuclear protein, is another regulatory gene believed to be involved in both differentiation and development of the immune system. Antisense RNA was employed to study the function of the c-fos gene upon class I MHC antigens. A c-fos antisense plasmid was prepared and introduced into F9 embryonal carcinoma cells. Such cells, which express c-fos antisense RNA, were unable to synthesize c-fos RNA and c-fos proteins in response to interferons and phorbol esters. The antisense c-fos clones also showed a reduced expression of the c-myc oncogene. A novel factor, which may be involved in a global gene activation process at birth, was found to be specifically expressed at the neonatal stage. The "neonatal protein" binds the enhancer region of the c-fos gene, which may be involved in increased expression of class I MHC genes after birth.

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Developmental Endocrinology Branch

D. Lynn Loriaux, M.D., Ph.D., Chief

Left to right: F. Cassorla, G. Cutler Jr., D.L. Loriaux, B. Nisula, G. Chrousos

The goal of the Developmental Endocrinology Branch is to enhance our understanding of the role played by the endocrine system in growth, development, and reproduction. The broad themes of this Branch's research derive from questions that emerge from the medical management of men, women, and children with disorders of the endocrine system, but a special effort is made to choose investigative problems that can only be addressed optimally using the unique resources of the NIH Clinical Center, the world's largest hospital devoted solely to research. Currently, problems in reproduction, growth, development and the endocrine responses to stress are under investigation. These studies are directed by **Lynn Loriaux, Fernando Cassorla, George Chrousos, Gordon Cutler**, and **Bruce Nisula**. The following summary will highlight emerging concepts, show how recent findings have altered our current understanding, and how results in this Branch have stimulated new or different lines of investigation.

Studies of reproduction in women have taken two lines: First, there has been an attempt to understand the endocrinology of the normal reproductive process, including pregnancy, and second, an attempt has been made to identify critical points in the process that will allow us to enhance fertility or suppress it in a safe, convenient, and reversible way. Studies directed at understanding the biochemical and physiologic mechanisms of the reproductive cycle in women have yielded several valuable results in the past year. The "prime mover" of the reproductive cycle is the episodic secretion of GnRH by the hypothalamus. The episodic bursts of secretion occur at a frequency of 90 - 120 minutes until late in the luteal phase when the frequency slows dramatically. It has been tempting to attribute disorders of the reproductive cycle to aberrations in the frequency of GnRH secretion. This group has tested this hypothesis by inducing reproductive cycles in women who have GnRH deficiency, with GnRH given by intravenous or subcutaneous injection at frequencies ranging between 60 and 180 minutes. All pulse frequencies induced cycles, but the incidence of ovulation was greater when GnRH was given at the 90 to 100 minute frequency. When ovulation occurred, all cycles were the same, regardless of pulse frequency. These studies suggest that the tolerance to variations in pulse frequency is great, that "frequency alterations" are not likely to be a prominent cause of infertility, and that the amplitude of the pulse is probably critical.

There has been considerable attention given to the "Premenstrual Syndrome" by the lay press. Because of their experience with the normal reproductive cycle, these workers were in a good position to examine the endocrine profiles of women having the premenstrual syndrome as defined by significant and reproducible changes in "affective" symptoms in the five days preceding menses. Hormones were measured every other day in the first half of the cycle and everyday in the second half. Plasma levels of estradiol, progesterone, cortisol, testosterone, and prolactin, among others, were not different between affected and non-affected women. These findings suggest that premenstrual alterations in mood cannot be attributed to alterations in hormone profiles, and raise questions as to the rationale underlying the current widespread use of progestins to treat the premenstrual syndrome. These investigators have examined the efficacy of one such regimen in a prospective placebo controlled trial. Progesterone suppositories were given for the seven days preceding the expected time of menses to women with or without premenstrual symptoms. Preliminary review of the data has revealed no difference in "affective" scores between the progesterone and placebo treated groups. This finding supports the notion that the premenstrual syndrome does not have an endocrine basis.

The availability of the progesterone antagonist, RU 486, has provided two opportunities to advance our understanding of the reproductive cycle: First, our current hypothesis regarding how the ovary signals the pituitary gland when a dominant follicle is ready for ovulation is that a "progestin" signal is sent by the ovary and read by the pituitary gland. This hypothesis can be tested using the progesterone antagonist RU 486 to interrupt the signal. If the hypothesis is correct, ovulation should be delayed. Women with hypothalamic amenorrhea, i.e., GnRH deficiency, were given pulsatile GnRH therapy to induce normal reproductive cycles. RU 486 or a placebo was then given 2 days before the expected time of ovulation and the effect on the timing of ovulation was measured. RU 486 delayed the ovulatory surge of gonadotropins. This supports, but does not prove, the concept that progesterone plays an important role in the transmission of the "readiness" signal from the dominant follicle. It also provides a new rational strategy for modulating the "ovulatory" event. Second, the idea of continuous antagonism of the progesterone effect on endometrial function as a "contraceptive strategy" can be tested. Early studies are encouraging. Marked maturational delay of the endometrium was caused by RU 486 in very small amounts, 1 μg/kg/day, or about 1/5000 that of the currently recommended dose for gestational interruption. These findings support the idea that low grade antagonism of the progesterone effect may offer a new, effective, and safe approach to contraception.

Improved understanding of the process of implantation and its transition into early pregnancy has been a natural derivative of the Branch's interests in reproduction. The examination of this process has concentrated on the study of human chorionic gonadotropin. Recent studies have yielded significant advances in our knowledge of the structural, functional, and metabolic properties of human chronic gonadotropin (hCG) and other hCG-related molecules prevalent in pregnancy. While pregnancy urine contains appreciable amounts of hCG, hCG β-subunit, and hCG ∝-subunit, the hCG-related molecule in greatest abundance is a fragment of the hCG β-subunit known as the β-core. At certain stages of pregnancy, β-core accounts for more than 99% of the immunoreactive hCG in urine. Little is known, however, about the structure of this important molecule. In the past year, β-core has been purified from pregnancy urine and its peptide composition and carbohydrate structure characterized. A major obstacle to clinical research on the β-core fragment of hCG has been the extensive cross-reactivity of hCG, hCG β-subunit, and LH in the available assays. To circumvent this problem, these investigators have succeeded in developing a sensitive and specific radioimmunoassay for β-core. The concentration of β-core in the urine of pregnant women ranged between 10 and 4000 μg/L. The urine of non-pregnant women contained less than 5 μg/L. Urinary β-core concentration was also elevated in five men with breast cancer. In one patient, urinary β-core was elevated despite the absence of detectable serum hCG. These findings suggest that β-core may become a valuable cancer marker.

The study of human growth has been a second focus of activity, and the optimal approach to the diagnosis of growth hormone deficiency has been under examination. The measurement of plasma growth hormone concentration every 20 minutes for a 24 hour period is currently in vogue as a diagnostic test. The mean concentration of growth hormone and its secretory pattern can be established from these measurements. Other investigators, using this approach, suggest that the diagnosis of growth hormone deficiency can be made on the basis of these two parameters. These workers have examined this hypothesis and their data does not support the conclusion. They have shown that the normal range of these parameters is so great that any mean "abnormal" level of growth hormone or any "abnormal" pattern of growth hormone secretion is encompassed within the range of normal. These studies suggest that a new approach to the diagnosis of growth hormone deficiency is needed, and also suggest that this approach is not likely to be based on tests currently employed for this purpose.

The treatment of growth hormone deficiency with growth hormone releasing (GHRH) hormone has been examined. Earlier studies showed that about 85% of children with growth hormone deficiency respond to GHRH, suggesting that most children could be treated with this agent if it could be shown to be effective in stimulating growth. This group has now shown that most children can be treated successfully, in the short term, with 10 μg/kg GHRH administered once daily as a subcutaneous injection. These findings illustrate that at least one alternative to growth hormone therapy, besides the hormone per se, exists for the treatment of growth hormone deficiency.

The pubertal "growth spurt" is known to depend upon sex steroids and upon an adequate plasma concentration of growth hormone. The precise way in which sex steroids modulate the accelerated growth of puberty is unknown. These investigators have shown that estrogens alone and in a very small dose can stimulate maximal growth of the epiphyses. Androgens can also stimulate epiphyseal growth, but it is unknown whether or not androgen stimulated growth is due to the androgens themselves or to the estrogens derived from the androgen precursors. Using the rat as a model, this group has now shown that the direct administration of androgen into the epiphyseal growth plate can stimulate growth, but only at very high dose. These findings support the idea that androgens must be converted to estrogens to induce epiphyseal growth. Accelerated growth is one of the serious untoward consequences of precocious puberty. To alleviate this problem, the improved diagnosis and treatment of sexual precocity has been given priority in the work of the Branch. An insight into the pathophysiology of familial male sexual precocity was gained in the past year. These investigators have examined the effect of sera from these patients on testosterone secretion from adult male cynomolgus macaque testes in vivo. In these monkeys, endogenous gonadotropin secretion has been inhibited with a potent luteinizing hormone-releasing hormone (LHRH) antagonist. Sera from patients with familial precocious puberty stimulated greater testosterone secretion than sera from normal prepubertal children matched for gonadotropin level. Although the difference is not yet statistically significant, these data suggest a potential molecular explanation for the disorder, i.e., a gonadotropin with a variant and "more potent" structure.

The treatment for gonadotropin-independent sexual precocity has been very unsatisfactory. This group has explored the use of an antiandrogen for the purpose. The antiandrogen spironolactone decreased the androgenic manifestations of precocious puberty, such as acne and spontaneous erections, but did not control accelerated growth and bone maturation. When the aromatase inhibitor testolactone was added, the accelerated growth was controlled. This finding again highlights the importance of estrogen in regulating linear growth. The results in the first nine patients to receive the combination appear favorable; growth rate and the rate of bone maturation were normalized. Studies with each agent individually have shown that the combination of spironolactone and testolactone is superior to either agent alone. The favorable results of this short-term study have encouraged the group to begin a long-term pilot study to attempt to normalize the growth rate, bone maturation, and the adult height of these boys. The approach has also been used to treat patients with the McCune Albright syndrome. In this case, only the aromatase inhibitor testolactone was used. This approach to treatment has been more successful than expected; inhibition of ovarian aromatase by testolactone has interrupted the process of ovarian cyst formation. Thus, the drug not only blocks estrogen formation by ovarian cysts, but actually prevents cyst formation and shrinks ovarian volume. Since the first publication of these results, the number of subjects under treatment has increased to 20. This should be a large enough group to begin to address the issue of how the treated children will compare to normal children in growth rate, bone maturation and, ultimately, adult height.

The Developmental Endocrinology Branch has pioneered the use of LHRH analogs for the treatment of gonadotropin dependent sexual precocity. The oldest of their LHRH analog-treated children have reached the normal age of pubertal onset and treatment has been withdrawn. The data from the first year in which the LHRH analog has been discontinued have now been analyzed. Gonadotropin secretion returned to normal between 3 and 12 months after stopping treatment. Pubertal progression resumed at the normal rate of approximately one Tanner stage per year. Growth rate after treatment has been appropriate for bone age. Thus, the available data suggest a prompt return of normal hypothalamic-pituitary-gonadal function after discontinuation of long-term LHRH analog treatment of central precocious puberty. The youngest children entered into this study have now been treated for more than six years. Puberty, growth rate, and bone maturation have remained suppressed during the entire period. The predicted adult height for this group (20 girls, 7 boys) has increased form 4'10" to 5'5". Thus, the current data from this large, long-term project remain very favorable.

The adrenal gland undergoes a series of maturational changes and plays an important role in growth and development. The DEB has several projects in progress concerning the physiology and pathology of the pituitary-adrenal system. The intractable hypercortisolism associated with the ectopic ACTH syndrome or adrenal carcinoma was successfully treated with RU 486, which has anti-glucocorticoid properties. RU 486 was also used to establish the manner in which glucocorticoids affect the immune response, evidence being obtained that these effects are glucocorticoid receptor-mediated. Using a clinical test based on the administration of ovine corticotropin releasing hormone (CRH), diagnostic refinements have been achieved to differentiate, on an outpatient basis, between Cushing's disease, ectopic ACTH syndrome and the hypercortisolism associated with depression. Also using CRH, it has been possible vastly to improve the treatment of pituitary Cushing's syndrome, which is caused by ACTH secreting adenomas. After CRH stimulation the adenoma is directly located by pinpointing the site of ACTH secretion; the surgeon can thus perform a ipsilateral hemipituitectomy, leaving no pituitary deficiencies. CRH is also helpful in differentiating between pituitary and hypothalamic causes of adrenal insufficiency. In vitro studies are yielding insights into the control of CRH release by the hypothalamus and placenta, and into the molecular biology of glucocorticoid receptors.

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Laboratory of Comparative Ethology

Stephen Suomi, Ph.D., Chief

Left to right: D. Symmes, F. Pedersen, J. Newman, S. Suomi, M. Bornstein, M. Lamb

The Laboratory of Comparative Ethology (LCE) carries out a program of research directed toward the study of behavioral and biological development in humans and in nonhuman primates. The influences on developmental processes of both genetic and environmental factors--and their multiple interactions--are explored in a comparative approach in order to determine the origins, ontogeny, and evolution of various behavioral phenotypes. Longitudinal designs are employed to address issues of ontogenic continuity vs. change, and a variety of both behavioral and biological measures reflecting multiple levels of analysis are collected concomitantly in investigations of developmental processes. A major emphasis is placed on characterizing and understanding normative patterns of biobehavioral development so that deviant patterns can then be readily recognized and their consequences evaluated with respect to established norms. Experimental results in nonhuman primates are correlated with the results of longitudinal studies of human infants and their families, as well as with results obtained by various neuroscience techniques.

During the past year, the Comparative Behavioral Genetics Section, directed by **Stephen Suomi**, continued to pursue broad-based investigation of individual differences in biobehavioral response to environmental challenge among rhesus monkeys, with special emphasis on characterizing the genetic and environmental factors that influence the development of such differences. First, standardized neonatal measures predictive of individual differences emerging later in development were refined and their use extended. Of particular interest was the finding, replicated on 3 independent groups of subjects, that relative amounts of quiet sleep, agitated sleep, and awake/alert states during the first month of life predicted individual differences in adrenocortical, behavioral, and immunological responses to stress throughout infancy and early childhood. Rhesus monkeys who as neonates spent significantly less time in both quiet sleep and awake/alert states and more time in agitated sleep subsequently displayed the highest levels of ACTH, the greatest incidence of disturbance behavior, and the lowest lymphocyte/neutrophil ratios when exposed to novel stimuli or challenging situations during their first and second years of life. In fact, these neonatal state measures proved to be as accurate in predicting individual differences in subsequent response to challenge as the rest of the entire standardized neonatal test battery. These findings are of considerable practical importance, in addition to their theoretical interest, in that useful predictions regarding individual differences in response to environmental challenge later in life can apparently be obtained relatively easily and nonobtrusively from both nursery and mother reared infants without physically handling the infants or disrupting the mother-infant bond. It thus may be possible to obtain comparable neonatal activity state data nonobtrusively from rhesus monkey infants reared by their mothers both within complex social groups in captivity and in natural troops living in the wild.

The standardized neonatal test battery was also modified for use with chimpanzee infants in a collaborative study with the Yerkes Regional Primate Research Center in Atlanta, with clear-cut differences in reflex and orientation scores emerging between full-term and premature subjects. Detailed analysis of the orientation scores also revealed that all subjects were much more responsive to both visual and auditory stimuli that are social in nature (human face, human voice) than nonsocial stimuli (e.g., colored balls and rattles). In addition, close examination of visual orienting behavior suggested that chimpanzee neonates have an optimal focal distance about twice that of human infants, a finding that may well account for the apparent failure of chimpanzee infants to maintain prolonged eye-to-eye contact when held by caretakers, in marked contrast to human infants in the arms of competent caretakers.

A second series of studies completed in FY88 examined the long-term consequences of differential early rearing in a number of domains. In these studies rhesus monkey infants were either reared by their biological mothers in dyad cages throughout the first 6 months of life or hand-reared in the neonatal nursery for the first 30 days of life and then placed into small groups of like-reared peers. At 6 months of age both mother reared and nursery-peer reared monkeys were merged into larger social groups where they remained together until puberty. Thus, except for the first 6 months of life, both mother and nursery-peer reared monkeys grew up under the same environmental conditions. Neuroendocrine differences between these two groups were apparent during the first 6 weeks of life, with mother reared subjects having higher basal levels of growth hormone and lower basal levels of plasma cortisol than nursery-peer reared subjects. Equivalent patterns of body weight increases in the two rearing conditions provided no evidence for an increased risk for failure-to-thrive syndrome in nursery-peer reared infants, as had been suggested by other investigators. On the other hand, differences between mother and nursery-peer reared monkeys in response to brief social separation at 6 months of age were apparent for a variety of behavioral and physiological measures, although such differences largely disappeared when these monkeys were subsequently combined into large social groups. Nursery-peer reared subjects displayed greater behavioral disruption than did mother reared monkeys in response to the separation manipulations. Nursery-peer reared subjects also displayed greater hypothalamic-pituitary-adrenal responsiveness and higher levels of CSF MHPG than their mother reared counterparts, but there were no significant rearing condition differences in separation levels of other CSF monoamine metabolites or in a variety of measures of immune system responsiveness. Moreover, rearing condition differences in response to similar separations carried out at 18, 30, 48, and 60 months of age were much more limited in scope and degree. Statistically significant rearing condition differences obtained at these later ages were largely limited to measures of self-directed behavior and CSF levels of MHPG and the serotonin metabolite 5-HIAA.

A third series of studies focused on continuity in biobehavioral response to challenge across developmental epochs. Data collected in 1988 in ongoing longitudinal studies of rhesus monkey biobehavioral ontogeny continued to demonstrate strong developmental continuities in virtually every aspect of response to environmental challenge and to link such continuities increasingly to genetic factors. Individuals who exhibited extreme behavioral reactions to challenge early in life tended to do so again in adolescence and early adulthood, although the nature of the reactions changed substantially across developmental epochs. Thus, infants who vocalized most frequently at one month were the ones most likely to exhibit behavioral withdrawal during brief separations at 6 months of age -- and to display the most stereotypy during comparable brief separations in adolescence. Infants who displayed the highest levels of plasma cortisol during early separations were most likely to have the highest levels as juveniles and adolescents, even though the absolute levels of cortisol during brief separation declined with increasing age. Juveniles who had extreme CSF levels of MHPG, HVA, and 5-HIAA when assessed at 6 months of age continued to display extreme levels at 18 months despite significant declines in absolute levels of HVA and 5-HIAA.

Pedigree analyses of the patterns of developmentally stable individual differences continued to provide compelling evidence that these individual differences are highly heritable. Comparisons involving both paternal half-sibs living together and apart (and, in both cases, with no direct experiences with their common biological fathers), as well as cross-generational comparisons, consistently demonstrated reduced variance between same-aged half-sibs compared with unrelated age-mates, between different-aged half-sibs compared with unrelated controls, and between fathers and offspring compared with unrelated members of successive generations, on most of the various behavioral and physiological measures for which long-term developmental continuities have been demonstrated. More sophisticated pedigree comparisons involving blood group factors with established loci are currently underway.

All of the above-described studies demonstrating long-term stability of individual differences in biobehavioral response to environmental challenge involved rhesus monkeys of known parentage born into a captive colony and followed longitudinally under well-controlled laboratory conditions. In an effort to assess the generality of these various findings and to address questions regarding the adaptive significance of stable individual differences in response to environmental challenge, this group recently began collaborative studies with the Caribbean Primate Research Center (CPRC) utilizing their population of wild-born rhesus monkeys living in natural troops. Observations of these rhesus monkey troops during 1988 documented the frequent occurrence of "natural" sequences of repeated short-term mother-infant separations during the annual 2-3 month breeding season. Throughout the breeding season adult females frequently leave their family groups and enter into consort relationships with individual males that will keep them occupied and away from their offspring for 1-3 days at a time. During these consort periods the infants who have been "left behind" typically display separation reactions that closely resemble those reported in the laboratory separation studies. In particular, there appears to be a wide range of individual differences in the severity of the infants' responses to these "natural" separations. These observations clearly indicate that repeated short-term separations from mothers are normative events for wild-living rhesus monkey infants and juveniles, with at least as wide a range of behavioral responses to these "natural" separations as has been reported in the extensive laboratory literature.

A second collaborative study currently underway at the Cayo Santiago Field Station involves careful prospective tracking of the process of adolescent male natal troop emigration through longitudinal study of a cohort of 19 juvenile males from one representative troop at the field station. In addition to behavioral observations conducted throughout the year, annual measurements of physical growth and maturation, hormonal profiles, and psychophysiological reactivity are being obtained from these juvenile and adolescent males during their annual capture by veterinary staff for tetanus shots and TB testing. At this point in the prospective study, approximately half of the young males have already emigrated from their natal troop, so it is possible to compare emigrant with nonemigrant males on a number of variables. A striking finding to date is that personality factors, and underlying psychophysiological characteristics, provide the best means of differentiating emigrant from nonemigrant males. Emigrant males are less fearful and more exploratory prior to emigration than are their nonemigrant agemates, and these differences are reflected in lower and more variable heartrates in standardized reactivity tests for the emigrant males (the relevant adrenocortical data are still under analysis). In contrast, neither social dominance status of the mother nor relative physical and hormonal maturational status differentiates emigrant from nonemigrant adolescent males. This finding is of special significance in that it suggests that individual differences in a behavioral tendency of clear biological (adaptive) relevance are more closely related to individual personality/constitutional factors than those of physical maturation or family social status.

A third collaborative study, involving a wild troop that was captured and removed from Cayo Santiago in 1984 and subsequently moved into a 2-acre enclosure at the CPRC, was continued in 1988. In the previous year blood samples and behavioral observations were obtained on all members of this troop when they were captured and given standard veterinary examinations over a 1-day period, and this past year equivalent data were gathered when the monkeys were once more captured for their annual veterinary check-up. Preliminary analyses of the data collected to date suggest strong year-to-year continuities in individual response patterns. In particular, strong positive correlations were found between levels of plasma ACTH obtained in each year's samples, and between measures of self-directed behavior displayed following each year's capture. Thus, the preliminary results from this study of wild-born monkeys living in a natural troop are consistent with previous laboratory findings of stable long-term patterns of individual differences in response to environmental challenge.

Vocalizations recorded from the above wild-born rhesus monkeys when they were briefly separated from their social group during the annual veterinary examinations were subjected to detailed acoustical analysis in collaboration with the LCE's Unit on Neuroethology, led by **John Newman**. The rationale for this study was that individual differences in reactivity to the stress of social separation would be reflected in the character of their vocal behavior. The subjects were divided into 2 groups on the basis of blood ACTH values sampled within 30 min. after separation from their group. Ten measures of vocalization (duration, amplitude, frequency, change in amplitude of frequency over time, etc.) were generated by computer. A correlation analysis between each acoustic variable and ACTH group revealed that 2 measures, call duration and pitch instability, had correlations of 0.89 and probability values that approached statistical significance.

A second study of the possible relationship between adrenocortical response to brief separation and vocal patterns was also carried out by Newman's group in 1988. This study attempted to find physiological correlates associated with individual differences in the rate of isolation call production by adult squirrel monkeys during a standardized 15-min. social separation test. Plasma ACTH and cortisol were assayed in this group of animals on 2 separate occasions, once immediately after removal from the home cage, and 1 month later, after a second 15 min. period of social separation. Overall, there was a significant increase in cortisol and ACTH levels with separation, but neither of the 2 assays in either the home or separated condition differentiated between the monkeys divided into vocal and nonvocal groups. Heart rate samples were also collected via telemetry from 2 of the nonvocal monkeys and compared with samples from 2 robust vocalizers in the social separation paradigm. Ongoing behavior was also recorded. Mean heart rate did not differ significantly between the 2 groups. However, there was evidence for greater periodic variability in heart beat interval in the vocal group, suggestive of higher "vagal tone" in these animals.

Another project initiated by the Unit on Neuroethology during 1988 involved testing novel drugs with suspected clinical value in the treatment of anxiety, depression, and other behavioral disorders. The drug of primary interest was milacemide [2-(pentylamino)-acetamide], already shown to have therapeutic value in treating epilepsy in humans and to have low toxicity in animals. In the initial study, 8 adult male squirrel monkeys (4 shown to be reliable vocalizers and 4 poor vocalizers based on prior screening results) were administered doses of milacemide (100-400 mg/kg, i.m.) and tested in the social separation paradigm one hour after drug administration. Milacemide produced a selective, dose-dependent reduction in the isolation calling rate of the "reliable vocalizer" group, without affecting motor behavior, but it did not change the behavior of the non-vocal group. Newman et al. initially hypothesized that the principal mechanism of milacemide-related reduction in isolation call production was through a GABA-mediated pathway. However, since milacemide is also known to inhibit the activity of monoamine oxidase (MAO-B), they subsequently tested the same group of reliable vocalizers with L-deprenyl, a drug known to irreversibly inhibit action of the MAO-B enzyme in brain. Since they found a significant reduction in isolation call production at high doses (2.5 and 5.0 mg/kg), MAO inhibition cannot be discounted as a possible mechanism for milacemide's action on vocal behavior. A complimentary approach to investigating the effects of peripherally administered drugs on vocal production is to study the effects of vocal behavior of chemicals introduced directly to the brain. As an initial step in this direction, Drs. Winslow and Newman, in collaboration with Dr. Tom Insel (NIMH), investigated the effects of corticotropin-releasing hormone (CRH) and an antagonist (alpha-helical CRH) introduced into the cerebro-spinal fluid of adult male squirrel monkeys through cannulae implanted in the cerebral ventricles. CRH produced dose related increases in motor activity, but not the increase in vigilance. The antagonist administered alone increased aggressive behavior directed at the subject's reflection in a mirror.

A final set of studies carried out in 1988 in the Unit on Neuroethology was directed at identifying and differentiating heritable influences on vocal development in primates. Work involving comparisons of the behavior of the "gothic arch" subtype of squirrel monkey in Costa Rica with captive social groups of the same subtype originating from South America identified vocal characteristics common to both Costa Rican and South American groups, as well as other vocal attributes found only in the Costa Rican population. Other work analyzed the development of the isolation call of infant common marmoset twins. Twins separated from their parents called together on nearly the same pitch, producing a unique acoustic signal that was readily identified and distinguishable from the isolation calls of either twin alone. Analysis of the isolation calls from the adult members of this marmoset colony revealed that each adult was very stable in its calling behavior over weekly 15 min. separations. Related work analyzed the temporal fine structure inherent in the serial production of calls by separated marmosets. Both common and pygmy marmosets produced isolation calls that were grouped together in a sequence of 2-10 closely spaced units, with a significant positive correlation between call duration and interval to the preceding unit. However, in the pygmy marmoset intervals and durations increased with sequence position, whereas in the common marmoset the opposite rule was followed. This is the first demonstration in any nonhuman primate of a rule of temporal ordering in a complex vocal sequence, and it suggests a fine degree of genetic programming in regulating the vocal output of these species.

Parallel studies of vocalization patterns utilized by squirrel monkeys living in complex social groups were carried out in the LCE's Section on Brain, Behavior, and Communication, led by **David Symmes**, focusing on calls used in social contexts characterized by quiet affiliative and caregiving behavior. This past year Drs. Symmes and Biben recorded the vocal behavior of six infants born in their colony during the first 3 months of life, using longitudinal time series sampling with close-in videotaping. Infant vocal behavior during the first month was very limited, restricted to simple tonal or pulsed calls associated with nursing, but even at this early stage other adult female monkeys (including those carrying their own babies) and juvenile females in the group interacted with infants very frequently and in ways which appeared to influence cognitive development, including close inspection with facial approximation, tactile exploration, and vocal exchanges. Both mothers and these other females ("aunts") used a similar call type, the "coax call", but the acoustic details of the coax call were clearly different in the contexts of nursing and retrieval. Use of this call by aunts seems especially promising for the study of squirrel monkey vocal development because the first identified vocal exchanges involving infant monkeys are with aunts. Moreover, these "dialogues" with aunts appear to differ from those with mothers and to change developmentally as the infants grow and become increasingly independent of the mother.

Continued study during 1988 of vocalizations emitted by juvenile squirrel monkeys during bouts of active play revealed that the primary function of "play" vocalizations was to alert adult group members to be more vigilant when the young are absorbed in play. Play has been shown to be a risky activity in other primates, exposing the vulnerable young to predation. However, the protection afforded by adults monitoring such activity allows youngers to play with abandon and in large numbers and compensates for what would otherwise be a maladaptive activity where animals crash through the trees, vocalizing loudly and oblivious to predators. This finding is further evidence for both the importance of play and the degree and variety of indirect parental care in this species. This group also continued its collaborative project with Drs. H. and M. Papousek from the Max-Planck Institute of Psychiatry in Munich and with the LCE's Child and Family Research Section investigating the acoustical characteristics of preverbal vocal exchanges between human infants and their caretakers. This research is based on the recent finding that a melodic mode of communication (probably with a genetic basis) is employed by human mothers and fathers in interacting with the prelinguistic infant, as evidenced by recent cross-cultural studies of native Chinese and English speaking mothers. The data for these studies were largely processed on the lab's sound analysis system. The model provided by this collaborative enterprise is being actively examined at the animal level.

Interactions between human preverbal infants and their caretakers provided the focus of several other major studies carried out in the Child and Family Research Section, directed by **Marc Bornstein**, in 1988. One study investigated the conditional contributions of three domains of maternal activity, including interpersonal affective communication, stimulation of infant attention, and control over object-centered exchanges, to infant language, play and representational competence at 13 months. Naturalistic observations of relevant mother-infant behaviors were conducted in the home and examined in relation to infant language and play competence. Correspondences between infant language and play skills were examined for evidence of an underlying representational competence that might itself relate to conditional maternal activities. Independent associations were found between maternal encouraging attention and infant noun-comprehension, and between encouraging attention and infant representational competence. Two-way interactions between maternal activity domains significantly augmented explained variance in infant skills, in that maternal social stimulation was associated with increased language and representational skills in dyads where mothers, rather than infants, exerted most control over object-centered exchanges, whereas frequent sociability, in the context of frequent maternal encouraging attention, was associated with greater infant play sophistication.

A second major study was designed to replicate and extend these findings by focusing on the extent to which three maternal characteristics (age, employment status, and parenthood status) and type of substitute care experienced during the mother's employment can influence the observed relations between caregiver social and didactic stimulation on the one hand and infant social and cognitive competencies on the other. In this study several groups of primiparous mothers and their infants are being observed when the infants are 5 months old; the groups differ systematically in terms of the mothers' mean ages (under 20 years, between 20 and 30, and over 30), employment status (employed vs. homemaker), type of substitute care, and whether the child has been adopted or not. Mothers and infants are being videotaped in their homes in both structured and unstructured interactions with each other and with the substitute caretaker.

A third major study initiated by Bornstein et al. in 1988 has focused on cross-cultural comparisons of mother-infant interactions and caretaking traditions. The purpose of this project is to identify significant similarities and differences in the childrearing ecologies of Japanese, Israeli, and American infants. It is widely held that Japanese and Americans differ in prominent aspects of their psychological make-ups and that certain social and intellectual distinctions between members of these two cultures arise early in life. Similarly, previous studies on the nature of infant development in Israeli kibbutzim determined that many decisive aspects of infant care--particularly the close ties between infants and mother -- vary markedly from the American experience. Cross-cultural developmental studies have shown that such rearing differences typically have implications for infants' later cognitive and social behavior and performance. In the present project infants being raised in Tokyo, in urban Haifa, and in a traditional Israeli kibbutz are being compared with infants reared in New York. Each infant is being observed on two occasions, at 5 and 13 months, in the presence of its caretaker. At this point data collection for the Japanese sample is complete, and similarities and differences among Japanese and American infants and mothers have been assessed. In addition, relations among infants' activities within each culture have been evaluated and resultant patterns of relations between the two cultures have been compared. Finally, interactions between mothers and infants in each culture have been studied and patterns of interactions across the two cultures compared. These results will be used to identify activity and interaction patterns which are distinctive to these two disparate cultures as well as patterns which are similar between the two cultures and which may point to processes universal in early development. Data collection has not yet been initiated for the Israeli sample. The study promises to be of great theoretical interest because of known differences in Japanese and American children's preschool performance (for the Japanese-American contrast) and because the childcare arrangement on traditional kibbutzim violates what are often considered to be crucial aspects of infant care -- particularly the close ties between infants and their mothers (for the Israeli-American contrast).

Cross-cultural comparisons were also utilized in several studies carried out in the Section on Social and Emotional Development, directed by **Michael Lamb**, to characterize the ways in which developmental niches can be described by variations in physical ecology, social and parental attitudes and values, and how differences in these dimensions affect children's development.

In one study, Lamb et al. followed up previous research on the quality of attachment between infants and adults on Israeli kibbutzim. Infants were tested in Ainsworth's Strange Situation procedure for assessing attachment with their mothers, fathers, and metaplot (careproviders) when they were 11 to 14 months old. At age 5, data concerning the functioning of these children were obtained by measuring IQ and empathy and by obtaining reports from preschool teachers and new careproviders concerning their behavior in kindergarten and the peer group using the CCQ and Baumrind's Preschool Behavior Q-sort (PBQ). A significant discovery was that C-type (resistant) attachments were frequently found on Israeli kibbutzim with communal sleeping, but the long-term correlates of this "insecure" pattern had not previously been identified. This group found no significant associations between infant-mother and -father attachment classifications and indices of later child development, but infants who had B-type ("secure") attachments to their metaplot were later less ego controlled and more empathic, dominant, purposive, achievement oriented, and independent than C-group ("insecure/resistant") subjects. All these group differences were in the direction predicted on the basis of prior research on the correlates of infant-mother attachment. All the measures of socioemotional development reflected the children's behavior in the children's house but not at home or with their parents, a finding that may explain, in part, the relatively strong predictive power of attachment status with metapelet as opposed to attachment status with mother and father. These results underscore the central importance of the metapelet as a key figure in the early social life of kibbutz infants. The findings thus raise questions regarding the developmental significance of attachment relations with various significant adults.

Another major project carried out in 1988 involved analyses of data from a longitudinal study in Sweden examining the effects of center day care, family day care, and home care on the development of 145 children recruited at an average of 16 months of age. Multivariate analyses using Wold's Partial Least Squares "soft modelling" procedure indicated that type of care had no reliable impact on the children one and two years post-enrollment. The quality of care received at home and the quality of alternative care had the most consistent and equivalent impact on personality, maturity and emergent social skills with peers and adults. Measures of family social support networks, temperament, and child gender had more modest effects. PLS analyses also showed that quality of home care was the most important predictor of intellectual competence one and two years after enrollment. Compliance with maternal requests in a task-like situation was most strongly predicted by the quality of care received at home. The quality and extent of alternative care were also significant predictors of compliance. The significance of these findings lies in their emphasis on the need to consider not only the type but also the quality of out-of-home care, and to consider the role of factors outside the care setting--such as the quality of home care--when evaluating day care arrangements. Other work on this project involves a small but intensive study of family day care in Utah and an exploration of the association between day care and security of infant-mother attachment in nearly two dozen studies conducted by other investigators. Data for both projects are currently being prepared for analysis.

In a third study, Lamb et al. explored the effects of agreement between Swedish mothers and fathers regarding socialization values. Parental agreement was computed by correlating the responses of 128 mothers and fathers on Block and Block's Q-sort concerning the values and attitudes they bring to the socialization of their preschool-aged children. The children's functioning was assessed using the Griffiths Developmental Scales, and the Blocks' California Child Q-sort (CCQ), yielding a measure of perceived ego resiliency and ego control. Marital quality was assessed using the two parents' independent responses to the Areas of Change Questionnaire. Data analyses completed to date revealed substantial disagreement between spouses in a substantial number of areas, with mothers showing more expressive and fathers more instrumental concerns. There were few differences between the parents of girls or boys. Parental agreement was associated both with marital quality and with contemporaneous and earlier maternal reports of ego resiliency in both boys and girls, as well as with maternal reports of ego control in girls only. There were no significant correlations between parental agreement and measures of intellectual development in either boys or girls, however. The results suggest that parental agreement may have a less general and a less gender-differentiated impact on psychological functioning in contemporary Sweden than was true in the United States when the Blocks' data were gathered 20 years ago, when it was reported that the degree of parental agreement had a substantial impact on child development, especially among boys.

Major progress was also made in 1988 in ongoing studies of the antecedents, correlates and consequences of adolescent pregnancy and parenthood, utilizing data from two large nationally-representative samples as well as smaller samples. The goal is to describe the psychosocial context of adolescent parenthood and to explore the long-term effects for both mothers and fathers. Analyses completed this past year revealed that regardless of race, adolescent parenthood was found to be but one symptom of a wider variety of psychosocial problems. Compared with nonfathers and nonmothers of similar ages and backgrounds, adolescent parents, especially the males, were much more likely to have a history of involvement with the police, school problems, and substance abuse. A smaller study showed that adolescent fathers differed in their attitudes and expectations from adult fathers, and that adult fathers with adolescent partners resembled adolescent fathers more than adult fathers with adult partners. Additional analyses revealed that adolescent marriage was associated with deficits in marital stability, income, educational attainment, and occupational prestige through at least 40 years after the marriage. For mothers, both adolescent childbearing and adolescent marriage were associated with higher lifetime fertility, lower income, less prestigious occupational ratings, lower educational attainment, and more frequent marital dissolution. The "best" outcomes were obtained by those women who delayed both childbearing and marriage into adulthood. These findings strongly suggest that adolescent parenthood is not a random event. It may also have long-term effects on the psychological and socioeconomic status of both men and women.

Finally, studies completed in the Unit on Parent and Infant Studies, led by **Frank Pederson**, investigated the relationship between emotional factors during the pregnancy period, the infant's temperament, and postnatal parent-infant adaptation. The first of these studies examined reactivity patterns to recorded infant cries that differed in their degree of aversiveness. Expectant mothers, their spouses, and nulliparous married women were compared in order to examine effects of pregnancy status and sex of the respondent. Recordings of normal infant cries and cries that were deviant on the basis of spectographic and clinical criteria were presented to the 3 groups of adults, and the respondents filled out rating scales after each cry to indicate their subjective evaluation while their heart rate and vagal tone were recorded continuously. All three groups made clear-cut distinctions in the cries on the basis of their subjective evaluations, with the deviant cries consistently rated as more aversive. On the physiological measures, however, the expectant mothers did not discriminate between the aversive and nonaversive cries while the expectant fathers and nulliparous women did, consistent with the hypothesis that the discrepancy between cognitive/subjective and physiologic responses evident in the pregnant women may be adaptive during pregnancy, serving as a protective mechanism for the fetus.

A second study concerned infant individuality, as assessed in behavioral observations conducted in the laboratory, physiological measures, developmental tests, and parental reports of temperament in 3-month-old infants. Infants were classified into groups of high or low heart rate variability (vagal tone), a measure that in other studies has been implicated with underlying central nervous system functioning, maturity, and temperament. Infants with high vagal tone scored significantly higher on the Bayley Mental Developmental Index and showed more rapid visual habituation. In contrast, ratings of temperament, whether based on independent observation in the laboratory or parental report, did not discriminate the groups, although there appeared to be more congruence between observational ratings and parental reports in the high vagal tone group, suggesting that the validity of parental reports of temperament may be partly influenced by characteristics of the babies being rated. The relationship between vagal tone and habituation rate at 3 months is noteworthy because, in other research, both measures have been found predictive of later cognitive functioning.

A third study investigating the consequences of pregnancy loss focused on differential effects of early vs. late loss on a measure of grieving and psychological preoccupation with the loss, the Perinatal Bereavement Scale (PBS). Along with other procedures the PBS was administered to expectant mothers and fathers in the third trimester of a pregnancy that was within 2 years of a previous loss; procedures were repeated at 6 weeks postnatally and when the infant was 16 months old. Comparisons were made between families that had experienced an early loss (loss prior to the 20th week of pregnancy) and families that had a stillbirth or neonatal death. Preliminary analyses indicated that grief is greater for late loss than early loss parents, that mothers experienced greater grief as measured by the PBS than did fathers, and that grief over previous loss diminished with time after the birth of a viable baby. The analyses also revealed a significant 3-way interaction among these variables, in that late loss mothers showed greater grief at all time periods, such that by age 16-months, early loss mothers and fathers and late loss fathers were indistinguishable from each other, but late loss mothers still showed elevated grief scores.

A final study carried out in the Unit in 1988 investigated what parents teach pre-school age children about caregiving during the course of play with dolls. Preliminary results yielded relatively few differences related to sex of the parent but more differences in play behavior related to sex of the child. Both older boys and girls enacted more caregiving activities, e.g., feeding, bathing the doll, etc., than did younger children. Mothers and fathers were relatively similar to each other in eliciting play with dolls, but both parents verbalized different messages to boys and girls. Girls were more frequently told that the doll was "their baby" or that they were the doll's "parent." Girls, in turn, verbalized a parental relationship to the doll more often than boys did. Boys often played out a nurturant role while not articulating verbally that they were in such a role. The results suggest that nurturing the young is an internalized role script for both male and female children very early in life, but females receive and develop an overlay of verbal constructions to support such behavior.

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Endocrinology and Reproduction Research Branch

Kevin J. Catt, M.D., Ph.D., Chief

Left to right: M. Dufau, H-C. Chen, K. Catt, G. Aguilera, K. Huang, C. Strott

The research programs of the Endocrinology and Reproduction Research Branch are directed at the elucidation of cellular mechanisms involved in hormone secretion and action. These programs include studies on the characterization of peptide hormones and their cellular receptors; the structure-function relationships of peptide and glycoprotein hormones; the regulation of hormone biosynthesis and secretion; and the mechanisms of peptide hormone action in endocrine target cells. Of particular interest are the analysis of pituitary-gonadal and pituitary-adrenal regulation, the control of ovarian activity during the reproductive cycle and pregnancy, and the receptor-mediated control of pituitary, gonadal, and adrenal function. During the current year, research has been performed on the receptors and signalling processes that are responsible for the control of differentiation and secretion in endocrine target cells. The role of hormones in cellular regulation has also been examined in selected forms of normal and disordered human endocrine function, and in appropriate animal model systems for the analysis of hormone secretion and the stimulatory and inhibitory control of target-cell function. The staff of the ERRB share common interests in the mechanisms of action of peptide and glycoprotein hormones, the role of neuropeptides in hypothalamic-pituitary regulation and stress, the control of gonadal and adrenal function by pituitary hormones, the renin-angiotensin system and aldosterone secretion, and the mechanisms and roles of protein phosphorylation in metabolic regulation.

Previous findings on the role of guanine nucleotide regulatory (G) proteins in AII binding and action indicated that the adrenal AII receptor is coupled to Gi as well as to the functionally identified but as yet undiscovered protein (Gp) responsible for coupling of calcium-dependent hormone-receptor systems to phospholipase C. Direct evidence for the role of G nucleotides in the stimulation of polyphosphoinositide hydrolysis by AII was obtained in permeabilized, inositol-labeled adrenal glomerulosa cells. In such cells, nanomolar concentrations of AII stimulated $InsP_3$ formation within 15 sec., and similar but less rapid responses were elicited by guanine nucleotides and fluoride. In adrenal membrane preparations, GTPγS-stimulated polyphosphoinositide hydrolysis was enhanced by Ca^{2+}, with half-maximal activity at 300 nM free Ca^{2+}. Ins-1,4,5-P_3 formation was also increased by NaF, further indicating the involvement of a guanine nucleotide regulatory protein. In addition to Ins-1,4,5-P_3 and its metabolites formed during degradation via the 4-monophosphate pathway, AII and GTPγS stimulated the formation of the phosphorylated metabolite inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4-trisphosphate in permeabilized cells. The absence of a significant rise in inositol 1-monophosphate indicated that phosphatidylinositol hydrolysis was not stimulated by AII or GTPγS. Pretreatment of glomerulosa cells with pertussis toxin for 12 h before permeabilization did not inhibit AII- or GTPγS-stimulated inositol polyphosphate formation. However, treatment with cholera toxin, forskolin, or 8-Br-cAMP for 12 h enhanced both basal and ligand-stimulated Ins-1,4,5-P_3 production. These observations suggest that agonist binding to the AII receptor activates a polyphosphoinositide-specific phospholipase C in the adrenal glomerulosa cell, and that a distinctive guanine regulatory protein is involved in this mechanism.

Characterization and purification of the AII receptor has been pursued by two approaches based on detergent solubilization of receptors from the bovine adrenal cortex. The complete isolation of the AII receptor by conventional purification techniques has been hampered by its extreme instability, which prevents the use of ligand-affinity procedures employed to purify several other peptide hormone receptors. For this reason, purification of photoaffinity-labeled AII receptors was performed by ion-exchange, lectin-affinity, and immunoaffinity homatography, the latter employing solid-phase antibody specific for the N-terminal region of AII that is accessible in the hormone-receptor complex. Since this method did not provide sufficient quantities of receptors for sequencing, the rapidly-inactivating free AII receptor has also been purified from solubilized bovine adrenal membranes by rapid lectin and ligand-affinity chromatography. This method has been more successful in providing large quantities of the putative receptor protein, and promises to yield sufficient material to permit microsequencing of the receptor.

An additional approach to characterization of the AII receptor, as well as to the analysis of its activation mechanisms, has been to express the AII receptor from mRNA extracted from the adrenal glomerulosa zone and other AII target tissues. The expression of several neurotransmitter and drug receptors from injected exogenous mRNA in Xenopus laevis oocytes has been demonstrated by electrophysiological measurements on ion channel activation. The expression of specific receptors for peptide hormones in such a translation system would facilitate studies on the structure and regulation of cell-surface receptors as well as their coupling to membrane transduction mechanisms. The expression of receptors for calcium-mobilizing hormones in Xenopus oocytes was sought by analysis of phospholipid turnover in hormone-stimulated oocytes. For this purpose, Xenopus oocytes were injected with mRNA extracted from bovine adrenal and pituitary glands and incubated with *myo*-[^{3}H]inositol to label plasma-membrane phosphatidylinositol phosphates. The expression of functionally active receptors for AII and thyrotropin-releasing hormone (TRH) was demonstrated by the stimulation of [^{3}H]inositol phosphate production by AII and TRH in the mRNA-injected, [^{3}H]inositol-prelabeled oocytes. The ability of AII and TRH to act by way of newly synthesized receptors from mammalian endocrine tissues to stimulate phosphatidyl-inositol polyphosphate hydrolysis in Xenopus oocytes suggests a generalized and conserved mechanism of receptor coupling to the transduction mechanism responsible for activation of phospholipase C in the plasma membrane.

The Section on Endocrine Physiology (**Greti Aguilera**) investigates physiological and pathological aspects of circulatory homeostasis and neuroendocrine regulation, including mechanisms of adaptation to stress. This program also includes studies on the role of the renin-angiotensin system in the regulation of mineralo-corticoid secretion and blood pressure, and the effects of AII in other systems including the pituitary and gonads. AII has been shown to mediate the increase in aldosterone secretion during sodium restriction, but the adrenal effects of the peptide are dependent on the sensitivity of the glomulosa zone to AII. Previous studies in the rat have demonstrated that adrenal responsiveness to AII depends not only on the trophic effects of the peptide, but also upon the modulatory effects of other regulators including dopamine, atrial natriuretic factor (ANF) and somatostatin (SRIF).

Studies on the effects of the dopaminergic antagonist metoclopramide (MCP) in sodium-loaded hypophysectomized rats showed that the sensitizing effect of MCP on the adrenal response to AII is blunted in the absence of the pituitary gland. In addition, abundant receptors for AII, which undergo regulatory changes during altered sodium and during administration of AII and MCP, are present in the intermediate lobe of the pituitary. These findings suggest the involvement of the pars intermedia of the pituitary gland in adaptation to changes in sodium intake, and provide an additional mechanism whereby dopaminergic regulation of an intermediate lobe peptide could modulate the physiological changes in responsiveness of the adrenal glomerulosa zone.

Studies on the ontogeny of the AII receptor revealed dramatic changes in receptor concentration and tissue distribution during development. In addition to a marked decrease in AII receptor concentration in the adrenal capsule and smooth muscle with age, the fetal and neonatal rat and mouse were found to possess abundant AII receptors widely distributed in muscular and mesenchymal tissue throughout the body. Other components of the renin-angiotensin system were found in the fetus, suggesting a unique role of AII during development. Analysis of the role of the receptor-bound AII in adrenal glomerulosa cells revealed marked internalization of the agonist ligand following binding to receptors, but not of the bound antagonist ligand. In addition, there was significant accumulation of the internalized agonist in the nucleus, suggesting that AII has direct actions at the genomic level in addition to its recognized effects on plasma membrane transduction mechanisms.

In the pituitary gland, investigations were focused on the properties and regulation of the corticotropin releasing factor receptors and the mechanism of interaction between CRF and other regulators of ACTH secretion. In studies on the properties of the CRF receptor, gel electrophoresis analysis of detergent-solubilized CRF receptors crosslinked with ^{125}I-Tyr-oCRF indicated that the receptor is a single protein with a molecular weight of 67 kDa. The characteristics of the CRF receptor are similar in several different target tissues, including the anterior and intermediate lobes of the pituitary and the cortex, amygdala and olfactory bulb of the brain. Previous studies have shown that the down-regulation of pituitary CRF receptors that accompanies the increase in plasma ACTH following adrenalectomy is dependent on hypothalamic factors such as CRF and VP. In the rat, studies during stress showed transient increases in plasma ACTH during prolonged immobilization. The subsequent decrease in plasma ACTH in the continuous presence of stress is accompanied by CRF receptor down-regulation and desensitization of the pituitary. However, pituitary responsiveness in vivo as well as the potentiating effect of VP on CRF action in vitro are maintained, emphasizing the importance of the interaction between regulators during the physiological control of ACTH secretion. CRF receptors in the intermediate pituitary and brain are unchanged during chronic immobilization stress.

In studies on the mechanism of action of ACTH regulators, the synergistic effect of VP on CRF action was previously found to involve potentiation of CRF-stimulated cAMP production, suggesting that activation of protein kinase C is part of the mechanism of action of VP. Studies in isolated pituitary cells showed that inhibition of endogenous protein kinase C abolishes the effects of VP in the corticotroph. In addition, VP was shown to stimulate inositol phosphate formation in pituitary cells and to induce translocation of protein kinase C from cytosol to the membrane compartment.

The Section on Molecular Endocrinology (**Maria Dufau**) investigates the molecular basis of peptide hormone action, with particular emphasis on the characterization of gonadotropin receptors, activation of steroid biosynthesis in gonads and adrenal, and analysis of the biological activity of circulating gonadotropins. A major aspect of this program is concerned with the characterization of gonadal gonadotropin and prolactin receptors, and of the physical and functional relationships of the LH receptor site and adenylate cyclase.

The LH/hCG receptor from rat ovary and testis was purified by sequential affinity column chromatography and isolated as a single protein species on SDS-PAGE under reducing conditions. The purified testicular receptor was shown to be phosphorylated in vitro by the catalytic subunit of cAMP-dependent protein kinase. Occupancy of the receptors by hCG significantly increased the rate of phosphorylation by the catalytic subunit of cAMP-dependent protein kinase, while maximal stoichiometry of phosphorylation was not affected by hCG. However, preincubation of receptors with hCG for 30-60 min reduced the subsequent rate of receptor phosphorylation. Phosphorylation by protein kinase A did not affect the binding characteristics of the testicular LH/hCG receptor. The phosphorylated testicular and ovarian LH/hCG receptors bound effectively to hCG-Sepharose and wheat germ lectin, and when eluted were resolved as single bands of Mr 90,000 and 85,000 in SDS-PAGE. These studies indicate that occupancy by hCG leads to conformational changes which initially facilitate but subsequently reduce receptor phosphorylation, and that tight binding of hCG renders the phosphorylation sites less accessible.

In further studies on the differences in Mr of LH/hCG receptors from testis (90 kDa) and ovary (80-85 kDa), trypsin digestion of phosphorylated ovarian and testicular LH/hCG receptors showed six radioactive peaks with almost identical retention times. This finding suggests that the two receptors have homologous amino acid sequences and phosphorylation sites for protein kinase A, and that post-translational modifications are responsible for the differences in size of the testicular and ovarian receptors. Neuraminidase treatment of purified receptors caused reductions in Mr to 82,000 ± 3,400 (testis) and 77,000 ± 3,700 (ovary), and further treatment with O-glycanase had little effect on molecular size. However, endoglycosidase F caused a reduction in apparent Mr of the LH/hCG receptor, and deglycosylation with N-glycosidase and Endoglycosidase F produced a single labeled polypeptide of Mr=59,000 ± 3,000 for both receptors. These results indicate that LH/hCG receptors are sialoglycoproteins with predominately N-linked glycosylation, and suggest that changes in the glycosylation pattern could account for the size difference between testicular and ovarian receptors. The various enzyme treatments also suggested that the LH/hCG receptor contains sialylated N-linked carbohydrate chains of the biantennary and/or hybrid type.

Studies on the transduction mechanism of prolactin action were initiated in the Nb_2 lymphoma cell line, which is dependent upon lactogen for proliferation. The ability of cAMP to modify PRL-stimulated Nb_2 lymphoma cell mitogenesis, and differences between the effects of pertussis toxin and cholera toxin (i.e. biphasic effect, degree of inhibition) and also the differential effect of PMA on Nb_2 cell replication, suggests the involvement of one or more G proteins in PRL action or its modulation, including a cAMP-independent mechanism.

Previous studies in human and experimental animals have suggested that sex steroid hormones modulate the pituitary secretion of biologically active gonadotropin [i.e. decrease in the B:I (bioassay:immunoassay) ratio of LH after castration in rats is attenuated by androgen replacement; in human menopause/or gonadal failure increased B:I ratios are reversed by E_2 administration; young men have a higher B:I ratio than cycling women]. To examine the role of E_2 in modulating biologically active LH secretion, bioactive LH release was examined in men subjected to steady state E_2 infusion, and the feedback actions of endogenous E_2 on spontaneous and exogenous GnRH-stimulated pulsatile bioactive LH release were analyzed. Steady state intravenous infusion of estradiol at a dosage that mimics its endogenous production rate preferentially suppressed mean circulating bioactive LH concentrations, with a consequent significant decline in the plasma bio/immuno LH ratio. Conversely, antiestrogen treatment enhanced spontaneous bioactive LH pulse frequency, increased bioactive LH pulse amplitude, and augmented plasma intrapulse and interpulse bio/immuno LH ratios. Low-dose pulsed injections of exogenous GnRH also increased plasma bio/immuno LH ratios. However, tamoxifen attenuated the ability of exogenous GnRH to further enhance the bio/immuno LH ratio, suggesting that endogenous LH release was already maximally enriched in LH bioactivity during antiestrogen administration. The ability of estradiol to modulate specific properties of the LH pulse signal as well as its frequency may have significant implications in relation to pituitary function, and may also reflect direct actions of estradiol on the gonadotrope with effects on cellular processing and/or terminal glycosylation of LH molecules.

In earlier studies on GnRH-induced LH release in vivo, a single large bolus of GnRH and/or continuous GnRH infusions did not cause any change in the plasma bio/immuno LH ratio. However, it was recently observed that two consecutive submaximal pulses of exogenous GnRH resulted in an immediate and preferential release of bioactive LH, with a consequent increase in the plasma bio/immuno LH ratio. These findings might be explained by functional compartmentalization of releasable LH pools, such that pharmacological GnRH stimulation increases secretion from all pituitary LH pools to yield an integrated bio/immuno value, rather than selective release from a highly bioactive pool. Also, during continuous GnRH infusion there may be lack of definition of a small early pool (presumably of high bioactivity) and/or a mixture with a late pool of reduced bioactivity. Thus, it is inferred that spontaneous LH pulses, putatively generated in response to endogenous GnRH stimulation of gonadotropes, exhibit a relative enrichment in biological activity, and this degree of enrichment can be modulated by estrogen action. Such observations suggest a critical role for estradiol in regulating the functional attributes of the pituitary-gonadal axis in man.

Studies on the dynamics of bioactive LH release in healthy older men (ages 60-75) revealed significant attenuation of the pituitary's capacity to release biologically active gonadotropic hormone. The diminution of bioactive LH release could be unmasked by maneuvers designed to enhance endogenous secretion of LH enriched in biological activity. Such evocative procedures consisted of mimicking endogenous GnRH action by consecutive intravenous pulses of low-dose (10 μg) exogenous GnRH, or by presumptively augmenting endogenous GnRH secretion with antiestrogen treatment. Some healthy older men exhibited evidence of neuroendocrine dysfunction, reflected by irregular bursts of bioactive LH release followed by transiently low plasma bio:immuno (B:I) LH ratios. However, mean basal plasma bioactive LH concentrations, B:I ratios, and spontaneous LH pulse properties (peak frequency, amplitude, duration, and enhanced B:I ratios within LH peaks) were not altered in older men. On the other hand, augmentation of bioactive LH secretion and enhancement of plasma B:I ratios by pulsed injections of exogenous GnRH were either significantly reduced or absent in older men. In addition, although tamoxifen increased bioactive LH pulse frequency in both age groups and facilitated exogenous GnRH action in some subjects, older men increased their 12-h mean bioactive LH concentrations, B:I ratios, and bioactive LH peak amplitudes to a significantly lesser degree than young men. In summary, young and older healthy men exhibit similar mean basal plasma bioactive LH concentrations and spontaneous LH pulse properties. However, pituitary bioactive LH reserve is markedly attenuated in older men challenged with either exogenous GnRH or antiestrogen. Accordingly, we conclude that healthy aging men manifest an impaired secretory reserve for biologically active LH release.

In the fetal rat Leydig cell, estradiol causes up-regulation of its receptor and induction of the late steroidogenic lesion at 17α-hydroxylase/17-20 desmolase that is observed in the adult Leydig cell. The ability of adult Leydig cells to respond to sustained gonadotropin stimulation with increased androgen production was previously found to be limited by an estrogen-dependent refractory state, with decreased activity of microsomal $P\text{-}450_{17\alpha}$ and decreased testosterone production. Such inhibitory regulation of the testis by endogenous estrogen was not observed in fetal life, due to a very low level of aromatization capacity, with lack of up-regulation and/or induction of testicular estrogen receptors by estradiol. More recent studies have revealed that higher doses or frequent treatment of fetal cultures with LH increase aromatase activity and consequent E_2-receptor-mediated action for the induction of gonadotropin-mediated desensitization in fetal cells. Resolution of fetal Leydig cells by centrifugal elutriation demonstrated that in addition to a predominant cell type with fetal characteristics, a small population of adult-like Leydig cells is present in the fetal testis, and that a functional adult-like population emerges from the fetal Leydig cells during gonadotropin treatment.

In related studies, the extent to which modulatory actions related to changes in $P\text{-}450_{17\alpha}$ mRNA levels could account for steroidogenic stimulation and desensitization was evaluated. For this purpose, a partial length rat $P\text{-}450_{17\alpha}$ cDNA clone was characterized and identified. The 1 Kb cDNA insert, displaying high similarity with the previously isolated $P\text{-}450_{17\alpha}$ cDNA sequences from human, bovine and porcine species, and containing the conserved tridecapeptide and heme regions and termination codon, was employed to examine the regulation of mRNA levels in adult animals treated with hCG and in cultured fetal Leydig cells. During low-dose hCG treatment, an early increase in mRNA levels was followed by a return to control values at later times, while higher desensitization doses caused a marked reduction in the mRNA at 24 h and minor recovery at 48 h. After estradiol treatment, fetal rat Leydig cells maintained in the presence of LH showed 70% decreases in $P\text{-}450_{17\alpha}$ mRNA levels and testosterone production. These studies suggest that gonadotropin stimulation and desensitization of $P\text{-}450_{17\alpha}$ dependent enzymes in the adult rat testis as well as estradiol-induced desensitization in fetal Leydig cells are related to levels of $P\text{-}450_{17\alpha}$ mRNA. These studies have also demonstrated that a short loop feedback control by products of the androgen pathway leads to marked reduction of $P\text{-}450_{17\alpha}$ mRNA in the Leydig cell. This finding rules out mechanisms based on the generation of an inhibitor protein acting at the translational level, or inactivation of $P\text{-}450_{17\alpha}$ by reactive oxygen-free radicals derived from breakdown of the interaction of pseudosubstrate (testosterone) with P-450 of oxygen (P450-oxygen-complex), proposed in earlier reports by others demonstrating the reduction of enzyme mRNA. It is extremely interesting that a hormone known to be trophic to its target cell has such a dual effect, causing increased mRNA levels at low doses but a subsequent major reduction at higher doses and hence steroidogenic desensitization. The above studies were made possible by the isolation of the cDNA for rat $P\text{-}450_{17\alpha}$, since a bovine cDNA probe was found to be unsuitable for use in the rat. It has been recognized that $P\text{-}450_{17\alpha}$ is present in the human and bovine adrenal and not in the rat adrenal, but only now with the isolation of rat cDNA and the use of an homologous probe was it possible to conclusively demonstrate the absence of adrenal message in this species.

Other studies using fetal Leydig cell cultures have demonstrated that Leydig cells are a site of β-endorphin synthesis in vitro and that testicular β-endorphin is under direct control of gonadotropins. Acute stimulation of Leydig cells by hCG can markedly enhance β-endorphin secretion, and these changes are not mediated by testosterone. In contrast, testosterone or its metabolites may exert a negative autocrine modulation of β-endorphin, since inhibition of steroid biosynthesis markedly increased basal and hCG-stimulated β-endorphin output (by 100-200%). In addition, β-endorphin did not affect testosterone production, and opiate binding was not detected on Leydig cells. Since we have demonstrated functional β-endorphin receptors and opioid inhibition of FSH-stimulated androgen binding protein production in Sertoli cells, the β-endorphin produced in the Leydig cell may have paracrine effects that contribute to the quiescent state of the testis from early life to sexual maturation and could also be involved in the modulation of seminiferous tubule function during adult life.

The Section on Adrenal Cell Biology (**Charles Strott**) investigates the physiology and regulation of adrenal steriodogenesis, by characterization of cellular steroid binding proteins and soluble factors which mediate steroidogenic responses to ACTH, and analysis of cellular mechanisms of cholesterol utilization in steroid biosynthesis. The Section is also interested in the development of adrenocortical zonation and the regulation of adrenal steroidogenesis, and is currently concentrating on two areas of research: 1) adrenocortical calmodulin, calcium- and calmodulin-binding proteins, protein kinase systems, and the post-translational modification of proteins; 2) purification, immunology, and functional activity of soluble and membranous adrenocortical proteins including steroid-binding proteins.

The steroidogenic action of ACTH can be separated into acute and chronic phases. The acute ACTH response (sec-min) occurs primarily at the level of the mitochondria in regulating the rate-limiting step in steroidogenesis, the conversion of cholesterol to pregnenolone. The chronic action of ACTH (hours) occurs at the level of the genome and involves synthesis of various steroidogenic enzymes and co-factors. Both the acute and chronic actions of ACTH are dependent on the cytoplasmic synthesis of protein in that both responses are blocked by cycloheximide. In addition, both the acute and chronic actions of ACTH can be mimicked by cAMP. Based on mutation studies performed with an ACTH-responsive murine adrenocortical tumor cell line (Y_1), as well as ACTH receptor studies involving various adrenocortical cell-types, it is now accepted that in the adrenal cortex ACTH stimulates membrane-bound adenylate cyclase activity which leads to an increase in intracellular cAMP and the activation of cAMP-dependent protein kinase followed by steroid synthesis. The role of other protein kinases in this process such as Ca^{2+}-regulated kinases, if such a role exists, is not well understood. No adrenocortical regulatory phosphoprotein has yet been identified, and there is no evidence that the P-450 cholesterol side-chain cleavage enzyme is regulated by phosphorylation-dephosphorylation. The guinea pig is used as an animal model to examine ACTH steroidogenic action for the reason that its adrenal gland is composed of an ACTH-responsive outer zone and an ACTH-unresponsive inner zone. In this model, adenylate cyclase activation and cAMP formation in response to ACTH are similar for the two zones, suggesting that in the inner zone a defect has developed beyond the formation of cAMP. When cAMP-dependent protein kinase activity was measured it was found to be significantly less in the inner zone than in the outer zone. The meaning of this finding, however, is unclear since the activities of Ca^{2+}-regulated protein kinases were also significantly lower in the inner zone than in the outer zone. It has been suggested that calmodulin, a protein that mediates certain intracellular actions of Ca^{2+}, may play an important role in ACTH-stimulated steroidogenesis. For this reason, the calmodulin system has been examined in this model and evidence was found for calmodulin kinase III activity and an endogenous substrate, similar to elongation factor-2 (M_r 100,000). This latter system is known to be hormonally regulated in the rat corpus luteum, and may also be a significant regulatory system in the adrenal gland.

The initial reaction of steroidogenesis is the cleavage of the cholesterol side chain by a specific cytochrome P-450 enzyme located on the inside face of the inner mitochondrial membrane. The resultant steroid, pregnenolone, is then metabolized by enzymes having extramitochondrial locations. Thus, pregnenolone must move out of the mitochondria, crossing the organelle's inner and outer membranes. Despite the significance of pregnenolone efflux from mitochondria, the process remains poorly characterized. This process is currently being investigated in the guinea pig adrenal cortex, in which a specific pregnenolone-binding protein (PBP) has been identified. Although PBP behaves as a M_r 58,000 globular protein on gel permeation chromatography, it migrates as a M_r 34,000 protein on SDS-PAGE. A polyclonal antibody has been generated against the purified 34 kDa protein. At all stages of purification, including the starting material, Western blot analysis of isoelectric focusing gels reveals a similar pattern of apparent microheterogeneity with pIs of 6.8, 6.6, 6.4, and 6.2. The major dilemma at the moment is to distinguish between microheterogeneity of a single protein and a co-purifying contaminant. Additional purification steps are under investigation, and an effort to generate an N-terminal sequence is also under way. Studies with the polyclonal antibody demonstrate that the PBP is present only in the soluble fraction of the adrenal cortex. Immunocytochemistry indicates that the PBP is most abundant in the outer, ACTH-responsive adrenocortical zone. The latter finding is interesting because the pregnenolone-binding activity is far greater in the inner, ACTH-unresponsive zone of the adrenal cortex. It thus appears that there could be active and inactive forms of the binding protein, and this phenomenon is currently under investigation. It is possible that the active/inactive forms and the microheterogeneity of PBP are related. Success with the N-terminal sequencing will resolve the problem of purity unless the PBP is composed of two non-identical subunits (it is probable that the PBP exists as dimer in its native form). Once the polyclonal antibody specificity has been ascertained, it will be used to isolate the PBP mRNA. The ultimate goal is to develop a cDNA clone for the PBP and to determine the complete amino acid PBP sequence.

The Section on Molecular Structure and Protein Chemistry (**Hao-Chia Chen**) conducts research on the analysis, synthesis, and structure-function relationships of biologically active peptides and proteins. This includes the identification and synthesis of unusual structures and sequences in amino acids and peptides, and the development of new techniques for peptide sequencing and synthesis. Of particular interest are the structural design, chemical synthesis, and modification of molecules important to reproductive and developmental biology. A major component of this project focuses on the role of carbohydrate structures in the subunit association and receptor interactions of human chorionic gonadotropin (hCG), employing chemically modified derivatives of hCG for receptor binding and target cell activation studies. The HF deglycosylated derivative of asialo hCG was found to bind with 2-5 fold higher affinity in corpus luteum homogenates and 5-10 fold higher in testis homogenates than hCG. When added to granulosa luteal cells or testicular minces, it caused no cAMP production at 0.1 microgram/ml and inhibited the production of cAMP by hCG. In recent studies, pure hCG-alpha subunit was specifically deglycosylated at Asn^{78} by an enzymatic procedure. The heterodimer formed by combination of this subunit with intact hCG-beta displayed similar potency to HF-deglycosylated hCG in the rat uterine weight assay and in cAMP and testosterone production in rat Leydig cells.

hCG-beta chains isolated from urines of 5 choriocarcinoma patients were compared with those from molar and normal pregnant women by SDS polyacrylamide gel electrophoresis and by Western blotting using anti-hCG-beta-COOH peptide antiserum. The sizes found in the choriocarcinoma patients were either larger or smaller than those from molar or normally pregnant women and should permit distinguishing these cases from the normal range. A triantennary oligosaccharide structure in choriocarcinoma hCG implies different mechanisms of post-translational processing in the malignant trophoblast.

Other parts of this project focus on partial or total synthesis of biologically important peptides which 1) incorporate unique structural features as functionally important probes or for defined linkages to proteins, 2) represent portions of the sequence of proteins and are useful for immunological investigations, 3) display agonism or antagonism through substitutions of amino acid residues or changes in secondary structures. As an example of this approach, specific toxicity has been programmed into ovine corticotropin-(oCRF) and human growth hormone (hGHRH) releasing hormones by way of derivatization with an active ester of ^{10}B-enriched 1-(2-carboxymethyl)-1,2-dicarba-closododecarborane after the standard Merrifield solid phase peptide synthesis. In order to prevent perturbation of biological activity, carboranyl-acetylation was directed to the alpha-amino terminus of oCRF or the introduced epsilon-amino group of Lys at residue 41 for GHRH.

The derivatives are fully active for their respective hormone-releasing activities, and the labelled oCRF was able to suppress the ACTH release system upon irradiation with slow neutron gas without affecting the releases of GH, LH and prolactin in a pituitary cell culture system. Neutron radiation experiments with the labelled hGHRH are now in progress.

In other studies on biologically active peptides, substitution of Ala at Gly^{13} and Gly^{18} within the magainin 2 amide sequence was performed in order to enhance the potential of an amphiphilic alpha-helical conformation as revealed by the circular dichroism. This manoeuver resulted in one to two orders of magnitude increase of antimicrobial activity over magainin 2 in a wide variety of bacteria. The importance of a free amino-terminus for the full activity of magainin was also established. In addition, the synthesis of a 27-residue peptide deduced from the mouse protooncogene *c-fos* with Tyr as the amino-terminus was accomplished. The peptide was conjugated to bovine thyroglobulin by diazotized benzidine through the phenolic function of Tyr to produce a complex suitable for immunization to raise antisera to the *c-fos* gene product.

The Section on Metabolic Regulation (**Kuo-Ping Huang**) studies the role of protein kinases and phosphorylation-dephosphorylation of proteins in the regulation of cellular functions. The regulation and hormonal control of glycogen metabolism, and the activities of glycogen synthase and phosphorylase kinase, are also studied. The receptor-mediated turnover of membrane phospholipids plays an important role in the regulation of many cellular functions. Inositol 1,4,5-trisphosphate is believed to trigger the release of calcium from an intracellular nonmitochondrial pool, whereas diacylglycerol activates protein kinase C to modulate numerous cellular responses. This signal-transduction pathway has been implicated in the regulation of cell growth, differentiation, gene expression, hormone and neurotransmitter release, cell-surface receptor function, and cellular metabolism.

Three major classes of protein kinase C isozyme were found to be highly enriched in mammalian brains. Using isozyme-specific antibodies to determine the cellular distributions of these enzymes by light microscopic immunocytochemistry, strong staining was revealed in neuronal somata and their dendrites and weak or no reaction in axons and astroglial structures. In the cerebellum, type I PKC antibodies stained the Purkinje cell bodies and dendrites, type II PKC antibodies stained the granule cells, and the type III PKC antibody stained both Purkinje and granule cells. In the cerebral cortex, all antibodies stained neurons resembling pyramidal cells and their apical dendrites in layers II to VI, while layer I was nearly devoid of staining. However, the various isozyme-specific antibodies revealed distinct laminar distribution patterns of the positively stained neurons, and the type III PKC-positive neurons exhibited a higher density than those of type I or II PKC-positive ones, especially in layer II of the cingulate (retrosplenial) and piriform cortices. In the hippocampal formation, both pyramidal cells of the hippocampus and granule cells of the dentate gyrus were stained by all PKC antibodies. Subcellularly, type III PKC appeared mostly in the cytoplasm of these neurons whereas type I and II PKC seemed to associate with the nucleus as well. In the olfactory bulb, both type II and III PKC antibodies stained the periglomerular and granular cells, and the latter also stained the mitral cells. The distinct cellular and subcellular distribution of PKC isozymes suggest that each isozyme plays a unique role in various specific neural functions.

Using PKC isozyme-specific antibodies for immunoblot analysis, heterogeneous distribution of PKC isozymes was demonstrated in various regions of monkey and rat brains, and type I PKC was most abundant in cerebellum, hippocampus, amygdala, and cerebral cortex. By immunocytochemical analysis, type I PKC-specific antibody showed strong reactivity in various types of neuron in the hippocampal formation, amygdala, cerebellum, and neocortex. In the hippocampal formation, granule cells of dentate gyrus and pyramidal cells of hippocampus were heavily stained. The relative levels of PKC isozymes in several areas of monkey cerebral cortex involved in visual information processing were determined by immunoblot analysis. While type II and III PKCs appeared to be evenly distributed throughout the areas, type I PKC formed a gradient of increasing concentration rostral along the cerebral cortex (occipital to temporal) and then to the entorhinal areas. Neurobehavioral studies have demonstrated that the perirhinal and entorhinal cortices of the temporal lobe participate more than the striate and prestriate cortices of the occipital lobe in the storage of visual representation and that both hippocampus and amygdala are important in memory formation. Since type I PKC is present at high levels in hippocampus, amygdala, and temporal lobe cortex, one can predict that the type I protein kinase C may be important for mnemonic function.

Types I, II, and III protein kinase C have been shown to be products of the γ, β, and α genes of this enzyme family, respectively. Incubation of the highly purified rat brain protein kinase C isozymes with trypsin (kinase/trypsin=100) under identical conditions resulted in a preferential degradation of the type I and II enzymes, whereas the type III enzyme was relatively resistant to tryptic proteolysis. Degradation of the type III enzyme by trypsin could be facilitated by the addition of Ca^{2+}, phosphatidylserine, and dioleoylglycerol; none of these components alone was effective. Limited proteolysis of the three protein kinase C isozymes generated distinctive fragments from each isozyme, indicating that each molecule has specific trypsin-sensitive sites. Tryptic digestion of the type III protein kinase C was used as a model to determine the effects of various modulators on protein kinase C degradation. While Ca^{2+} and phosphatidylserine together were sufficient to convert the type III protein kinase C from a trypsin-insensitive to a sensitive form, addition of dioleoylglycerol greatly reduced the Ca^{2+} requirement for such conversion. Among the various phospholipids tested for trypsinization, phosphatidic acid and phosphatidylserine were most effective, whereas phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol were the least effective. With the exception of phosphatidic acid, the order of effectiveness of these phospholipids for trypsinization of the kinase paralleled that for the stimulation of protein kinase C activity. The relevance of these proteolytic reactions to physiological responses was assessed with phorbol ester on rat basophilic leukemia cells 2H3, which contained both type II and III protein kinase C and showed synergistic interactions between Ca^{2+} ionophores and the tumor-promoting phorbol esters for histamine secretion. Immunoblot analysis with the isozyme-specific antibodies revealed that phorbol ester induced a faster degradation of the type II than that of the type III isozyme in these cells. These results demonstrate that the various protein kinase C isozymes have different susceptibility to proteolysis in vitro when tested with trypsin, as well as toward endogenous proteases in intact cells.

Rat basophilic leukemia cells (2H3) can be stimulated to secrete histamine by aggregation of the I_gE receptors either directly with antireceptor antibodies or indirectly with antigen when cells are primed with the appropriate antigen-specific I_gE. The secretory response of the parental 2H3 cells appears to require an increase in the intracellular level of Ca^{2+} and activation of protein kinase C based on evidence including the synergism between Ca^{2+} ionophore and tumor-promoting phorbol ester, and the inhibition of secretion by inhibitors of PKC. In 2H3 cell variants having reduced secretory response to antigen, the level of PKC isozymes was determined by immunoblot analysis and by chromatographic separation on hydroxyapatite. Among ten 2H3 cell variants tested, all contained normal levels of the type III PKC as compared to parental 2H3. Several mutants having reduced responses to antigen for histamine secretion were found to contain reduced level of the type II PKC. Analysis of the PKC substrates in 2H3 and type II PKC-deficient mutant cells by in vitro phosphorylation with a proteolytically activated PKC revealed that the two cell lines had similar substrates for PKC. Hence, the different physiological responses observed in the mutant cells may result from the deficiency of type II PKC. These findings suggest that the type II PKC is involved in the ligand-mediated secretory response in 2H3 cells.

Effects of phorbol 12-myristate 13-acetate (PMA) on the fate of protein kinase C in two mouse thymoma cell lines, which are either responsive (EL4) or unresponsive (IEL4) to PMA-induced interleukin-2 (IL-2) production, were investigated with polyclonal antibodies raised against rat brain enzyme. These antibodies immunoprecipitated completely the protein kinase C from both cell lines and detected mainly an 82-KDa protein by immunoblot analysis of the crude homogenates as well as the partially purified kinase preparations. PMA elicited a time- and dose-dependent redistribution of protein kinase C from cytosol to the particulate fraction and proteolytic degradation of the kinase from both cell lines. The dose of PMA required for half-maximum protein kinase C translocation and degradation was at least 5 times lower for EL4 than for IEL4. In the presence of 16 nM PMA the rates of protein kinase C translocation and degradation were faster in EL4 than in IEL4, and the half-lives of protein kinase C in EL4 and IEL4 were less than 5 min and greater than 2 h, respectively. Analysis of the tryptic fragments of the immunoprecipitated enzyme, previously phosphorylated in the presence of $[\gamma\text{-}^{32}P]ATP$, revealed minor structural differences between the protein kinase C from these two cell lines. In neither cell line did the PMA-induced degradation of protein kinase C result in accumulation of the Ca^{2+}/phospholipid-independent kinase (catalytic unit) as analyzed by immunoblotting and gel filtration chromatography. Thus, activation of protein kinase C through its proteolytic conversion to the effector-independent catalytic unit plays little role in IL-2 production. The role of protein kinase C translocation and degradation in the PMA-induced responses in EL4 cells in unknown. However, IL-2 production in EL4 cells was reduced when PMA-induced degradation of protein kinase C was retarded by exogenously added protease inhibitors.

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Laboratories of the Scientific Director

Arthur S. Levine, M.D., Gordon Guroff, Ph.D.

Left to right: K. Dixon, A. Levine, C. Patch

The section led by **Arthur S. Levine** is using the DNA virus SV40 in two projects, the elucidation of the mechanisms whereby DNA damage in mammalian cells is converted into permanent mutations (mutation fixation), and the role of growth factors in the regulation of mammalian DNA replication.

Chromosomal mutations are the underlying cause of most inherited diseases and many developmental abnormalities. Mutations can also lead to alterations in gene expression in somatic cells, leading to loss of the normal differentiated phenotype and ultimately to cellular transformation. In work directed by **Kathleen Dixon**, a shuttle vector, pZ189, has been developed which contains the SV40 replication origin and early region, so that it replicates like SV40 virus in primate cells; the pBR327 bacterial plasmid origin and replication functions, so that pZ189 can replicate in bacterial cells; the gene for ampicillin resistance to provide a selectable phenotype in bacterial cells; and the supF gene which codes for a suppressor tRNA and provides the target for mutagenesis. Thus, the vector (pZ189) allows one to induce mutations in the mammalian system, identify them in a bacterial system, and then sequence them. Both at the genetic and biochemical levels, the vector has proved very useful for investigating how DNA replication fidelity is maintained, how DNA damage caused by mutagenic agents is removed by cellular DNA repair processes, and how damage that remains in the DNA interferes with normal DNA replication and induces mutations. With this vector, mutations induced in vivo (cultured primate cells) by two different carcinogens, UV radiation and benzo[a]pyrene diolepoxide (BPDE), have been analyzed. The majority of the UV-induced mutants carry either single or tandem double base substitutions, mostly G·C -> A·T transitions. BPDE-induced mutations occur almost exclusively at G·C base pairs and about 30% are single base deletions. In collaboration with P. Munson (LTPB), mathematical modeling and statistical analysis have been used to demonstrate that there is a good correlation between the sites and frequencies of UV-induced mutations and the sites and frequencies of UV-induced DNA damage (in the supF target gene), if it is assumed that the DNA polymerase preferentially inserts A opposite UV-photoproducts in the template DNA. In the case of BPDE-induced mutations, the mutational spectrum is consistent with the specificity of BPDE adduct formation (almost exclusively at G). The dramatic differences in mutational specificity of these two carcinogens indicate that the observed mutations are a result of the specificity of the carcinogen used and not some unusual characteristic of the target DNA or the assay system. The pZ189 vector system can also be used to study DNA replication fidelity and mutation fixation in vitro. The SV40-based shuttle vector can be completely replicated in crude extracts prepared from monkey or human cells which are supplemented with purified SV40 T-antigen. In initial studies with this system it was found that the fidelity of replication was much higher than that observed previously for gap-filling reactions with purified DNA polymerases, suggesting that there are additional factors in the crude system that serve to enhance replication fidelity. Experiments have also been started to examine the replication of carcinogen-damaged templates and mutagenesis of those templates in the crude in vitro system. Preliminary results suggest that some damaged templates can become fully replicated in the in vitro system, as they are in vivo, and furthermore, that this replication is accompanied by mutation induction. If further experiments confirm that the types of mutations induced in vitro are similar to those induced in vivo, this should prove to be a very powerful system in which to elucidate the molecular details of mutation induction in mammalian cells. The shuttle vector system is also being used to investigate the role that inducible cellular responses play in mutagenesis (analogous to the bacterial "SOS" response to DNA damage). When mammalian cells are treated with carcinogens prior to transfection with the UV-damaged vector, the frequency of UV-induced mutations increases. Thus, there appear to be indirect as well as direct effects of DNA damaging agents on mutagenesis in mammalian cells, just as in bacteria. DNA sequence analysis of mutants induced in carcinogen-treated cells transfected with UV-damaged templates indicates that there are no major changes in the mutational mechanisms in carcinogen-treated cells. This indirect effect of DNA damaging agents can be mediated by a secreted cellular factor; conditioned medium from UV-irradiated cells can induce enhanced mutagenesis in untreated cells.

Miroslava Protic-Sabljic has found that pretreatment of mammalian cells with sublethal doses of carcinogens enhances the expression of transfected UV-irradiated CAT plasmids in repair-proficient but not repair-deficient mammalian cells, suggesting an enhancement in the excision repair capacity of pretreated cells. This enhancement is specific for carcinogenic agents since the tumor promoter, phorbol myristate acetate, had no effect on CAT inactivation in either untreated or UV-irradiated cells. The enhancing signal is not transferable by secreted cellular factors from carcinogen treated cells, implying that different pathways are involved in enhancement of mutagenesis and enhancement of cellular repair. To determine whether some of the DNA damage-inducible cellular factors are DNA-binding proteins, DNA band-shift experiments were initiated in collaboration with S. Hirschfeld (LDMI) using cell extracts active in in vitro repair synthesis. Preliminary results suggest that there are several DNA-binding proteins that are very abundant in cell-free extracts prepared from carcinogen treated cells. Some of these proteins bind to double stranded and single stranded undamaged or UV-damaged DNA and their binding is highly salt specific; experiments are in progress to determine their binding specificity and their role in the cellular response to carcinogen treatment.

Adenovirus 2 (Ad2)- and simian virus 40 (SV40)-transformed hamster embryo cells differ markedly in a number of phenotypic properties, including their potential for inducing tumors in hamsters. Both Ad2- and SV40-transformed cells are immortalized and readily induce tumors in immunoincompetent newborn syngeneic hamsters, but only SV40-transformed cells are highly oncogenic in both adult syngeneic and allogeneic immunocompetent hamsters. The reasons for the difference in the oncogenic potential of the Ad2 and SV40-transformed phenotypes remain elusive. Work done in this laboratory by **Cephas Patch** et al. has shown, paradoxically, that SV40-transformed cells, but not Ad2-transformed cells, secrete a powerful mitogenic inhibitor (MI) with a molecular weight of 24-40 kd. The MI secreted by SV40-transformed cells is inhibitory for several transformed and untransformed cell types, and exerts a reversible cytostatic, not cytolytic, action on untransformed primary hamster embryo cells. Studies on the kinetics of secretion have revealed that the rate of MI secretion by SV40-transformed cells increases as the cells approach confluence, suggesting that one function of MI is to regulate growth of the cells that secrete it. A variety of studies indicates that MI is different from TGFß, a mitogenic inhibitor of about the same size. Another putative growth factor under study is the product of the SV40 small t gene. Deletion mutations in this gene alter the spectrum of tumors induced by SV40. Wild type SV40 virus, when inoculated subcutaneously in Syrian hamsters, normally induces fibrosarcomas at the injection site. In contrast, subcutaneous inoculation with small t deletion mutants of SV40 frequently results in induction of abdominal lymphomas instead of subcutaneous fibrosarcomas. This type of tumor has been reported to be induced by wild type SV40 only after intravenous injection of the virus. A detailed characterization of cell lines derived from the abdominal lymphomas (carried out in collaboration with A. Lewis and M. Carbone, NIAID) has revealed that these tumors are derived from MAC2+ macrophages. Another small t deletion mutant of SV40 induced osteogenic sarcomas in about 10% of the animals that developed tumors. This result is very provocative: Although the cellular origin of osteosarcomas is unknown, these tumors contain many giant osteoclast-like cells; since osteoclasts and macrophages both belong to the mononuclear phagocytic system, this suggests that the tissue tropism of the small t mutants may be limited to certain types of mononuclear phagocytic cells, some of which are MAC2+ macrophages and some of which are osteoclasts. Both cell types enjoy a high proliferative capacity, possibly explaining why these cells are readily transformed by SV40 even in the absence of the SV40 t "growth factor."

Nerve growth factor, studied intensively for many years in the Section led by **Gordon Guroff**, is one of the best characterized of the large and increasing number of growth and differentiation factors now known. These factors act on a wide variety of cell types. NGF is required for the survival and ultimate development of the sympathetic and sensory nervous systems, and has been shown to have some action on the adrenal medulla, on several populations of neurons in the central nervous system, and on tumors arising from cells derived from the neural crest. The biochemical mechanisms of NGF activity are of substantial interest because an understanding of them may shed some light on cell development in general and, specifically, on the growth and differentiation of neurons and on ways in which the selectivity of neural connections can be achieved, and indeed, altered. NGF or factors similar to NGF may also be involved in the re-growth of damaged or severed neural tracts, which would be of great clinical significance. An understanding in molecular terms of the way in which differentiation factors, such as NGF, act to limit the growth of normal cells promises to provide crucial insights into the growth regulation of normal cells and the tumors which arise from them. In recent years as it has become increasingly apparent that many of the growth factors are related structurally and functionally to specific oncogenes, and that alterations in the biology of oncogenes is at the heart of the process by which cells become malignant.

This Section continues to study the intracellular mechanisms by which NGF alters the phenotype of its target cells and has made the following advances during the last year. (1) Motivated by the finding that NGF stimulates phosphoinositide turnover, the effect of NGF on arachidonic acid release was evaluated; increased release might be expected as a consequence of the increased phosphoinositide breakdown, but presently the data indicate that phosphoinositide turnover and arachidonic acid release are quite independent. (2) Phospholipase A2 treatment, but not treatment with other phospholipases, causes neurite outgrowth on PC12 cells; although this outgrowth does not exactly mimic that caused by NGF, it is inhibited by K-252a, a specific inhibitor of the actions of NGF on these cells, suggesting that the action of phospholipase is important in the mechanism by which NGF elicits neurites. (3) NGF, a differentiating agent, causes an increase in the activity of one S6 kinase and EGF, a mitogen, causes an increase in the activity of a different S6 kinase; this indicates that the functional properties of the S6 protein change depending on whether the cells are instructed to divide or to differentiate. (4) Under certain circumstances, pertussis toxin inhibits some of the actions of NGF; this suggests that a G protein is involved in some of the actions of the factor. These results support the hypothesis that the combination of NGF with its receptor initiates several chains of phosphorylative actions, and that these phosphorylations are a major, if not the major, mechanism by which NGF influences the development of its target cells. Studies of the changes in the expression and function of different gene products, underlying the changes in cell structure and function caused by NGF, have led to the following advances during the last year. (1) Treatment of PC12 cells with dexamethasone prevents the normal NGF-induced increase in Ng-CAM, the neuron-glia cell recognition molecule; this manipulation leads to a population of apparently normally differentiated neuron-like cells that lack this important cell surface determinant, and may not attach to appropriate glia. (2) The decreased binding of EGF to cells treated with NGF appears to be due, not to a decrease in receptor synthesis, but to an increased phosphorylation of the receptor at the membrane.

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